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A Study of Equine Fistulous Withers and Poll Evil

A Complex Infection with Actinomyces Bovis and Brucella

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THIS PAPER deals with some observations made in a fundamental study of fistulous withers and poll evil of horses. Except for the difference in anatomic location, the configuration of the lesions, and the difference in the frequency of occurrence, the two conditions are essentially the same from the standpoint of etiology and pathology. In order to clarify the confusion in the etiology and pathogenesis of the condition, this study was undertaken from a four-fold approach: anatomic, bacteriologic, pathologic, and surgical.

THE ANATOMY OF THE SUPRASPINOUS BURSA

The ligamentum nuchae is the elastic portion of the supraspinous ligament and is capable of supporting the head and neck. The change from collagenous structure of the lumbodorsal portion of the supraspinous ligament to elastic fibers is gradual and takes place over the withers. Here the ligament loses its attachment to the thoracic spines. At the withers, the ligament reaches its maximum width of approximately 6 in., forming a saddle or cap which assists in maintaining its position. The funicular portion of the ligamentum nuchae is a direct continuation of the lumbodorsal portion but differs from it in that it is elastic and divided in the median plane into symmetrical parts. The bilaterally symmetrical lamellar portion of the ligamentum nuchae is attached to the funicular part and to the sides, below the summit, of the second thoracic spine. This leaves a small triangular space between the funicular and lamellar parts of the ligament and just anterior to the second thoracic spine. Williams and Frost[1] call this space the fenestrum, Ellenberger and Baum[2] mention it as a space or lake, while Sisson[3] does not mention it. The space contains a little fat and areolar tissue. It will, henceforth, be referred to as the fenestrum. At the withers, the trapezius muscle is attached to the deep surface of the saddle and the rhomboideus muscle to the thoracic spines by means of the dorsoscapular ligament.

The supraspinous bursa (bursa cucullaris of Ellenberger and Baum) is ventral to the ligamentum nuchae in the region of the third and fourth thoracic spines. Sisson and Ellenberger and Baum state that it may extend to the second or to the fifth spine. Sisson leaves one with the impression that the bursa is limited to the summits of the spines. Ellenberger and Baum state that it may extend laterally, or nearly, to the margin of the scapular cartilage. Lateral extensions of the bursa must lie between the trapezius and rhomboideus muscles. Sisson does not discuss the structure of bursae

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except to describe them, in connection with various muscles, as synovial bursae.

In the studies here reported, the positions and relations of the bursa were investigated by means of injected specimens using warm, thick gelatin colored with gentian violet. The injections were made on the dead animal, and then the entire region was removed and placed in the refrigerator over night. Dissections were made on the cold specimen in which the gelatin had set to a firm mass. In all, 14 specimens were examined. In specimens where the injection was completely successful, a delicate thin-walled sac distended with gelatin was found. The smaller sacs had uniform rounded borders, while the larger ones were more or less lobulated. Histologic examination of two bursae, while not completely satisfactory, revealed that the sac is lined with endothelium.

No two of the bursae examined were identical. The smallest and simplest of the bursae were rounded sacs about 5 cm. in diameter, located above the second to the fifth spines but not involving the entire area. Lateral migration between the trapezius and rhomboideus was the most common variation. These were both unilateral and bilateral. They may be symmetrical or diagonal and may or may not be connected by an isthmus over the spines. The largest of the laterally placed bursae reached the margin of the scapular cartilage. One bursa extended into the fenestrum and also pouched upward between the halves of the ligament forming a subcutaneous pocket.

The majority of the bursae had a diameter of 5 to 6 cm., but the largest measured 11 cm. long by 5 cm. wide. The capacity varied from 30 cc. to 90 cc. The outstanding fact of this phase of the investigation is the close correlation between the positions of normal and of diseased bursae.

OCCURRENCE

Several observers[4,5,6,7] have pointed out that poll evil and fistulous withers are essentially rural, rather than urban, maladies, although some of those clinical observations may have had little or no technical laboratory investigation to support that observation. Other workers have attributed the greater rural frequency of this characteristic condition to association with cattle and the prevalance of *Brucella abortus* infection in cattle herds. The etiology advanced in this paper is quite consistent with the clinical observation that horses in most cases have acquired the infection from association with cattle, which are the primary reservoirs of the infection, and supports the assumption that the condition is primarily rural in its occurrence.

PATHOLOGY

One of the significant features of fistulous withers is that there are so many variations in the arrangement, size, location, and anatomic relations of the lesions. No two cases of fistulous withers are alike. The only satisfactory way to study the gross pathology of the process is with unopened cases, because the secondary suppurative and proliferative changes following operative intervention obscure the primary pathologic process.

A group of 9 horses with fistulous withers, including one with poll evil in addition, were accordingly killed. With the withers in the normal erect position, a careful dissection was made of the region to observe the anatomical relations. It was possible in that manner to isolate and photograph the lesions, study their relationships, and remove them from the carcass for bacteriologic examination under optimum conditions.

From these studies, it appears that fistulous withers is primarily a bursitis. The early lesion is a simple thin-walled sac distended with a viscid ochre-colored serous exudate. This exudate arises in the course of the inflammatory changes in the inner layer of the bursal sac. Here are found the infectious agents involved in the active pathologic process. The fibrin increases as the process progresses, so that with coagulation the masses and flakes of fibrin give the exudate its characteristic appearance. There is often some blood in the exudate. Much fibrin is loosely adherent to the lining of the sacs. It is not a true phlegmonous process. Fistulous withers and poll evil are

identical in their general pathology. The later stages involve considerable necrosis. The variations in size and location of the lesions probably can be explained in the beginning by differences in the contour of the supraspinous bursa and the direction in which the distensions are formed. When the bursa pouches out from under the ligament, it usually is found under the trapezius muscle. The bursa may pouch upward between the lateral portions of the ligament. These two paths are the main channels of least resistance. The sacs increase in size by distension and dilatation from within as pockets of the fistulous sac are forced between the tissue layers.

The variations in the size, shape, and position of these saccular lesions are innumerable. In one case, the sac had a length of 20 in. It was adherent to the scapula but was not attached to the ligament. These sacs may extend well down over the scapular cartilage. In another old case, there was complete separation of the ligamentum nuchae with the necrotic ends embedded in the wall of the sac. The thickness of the tough connective tissue wall of the sacs varies from the thin distended portions about to rupture to nearly an inch. The pockets in advanced cases open into other sacs through small openings and the bilateral sacs may communicate under the ligamentum nuchae. While some local adhesions of the outer surfaces of the sacs are present, large areas can be separated and dissected from the adjacent muscles and fascia. Extensive necrotic changes in the ligament and occasionally in the dorsal spines represent well-advanced stages of the pathologic process, and are not likely to be encountered when the bursal sac has distended laterally for some distance from the ligament.

The microscopic pathology reveals considerable uniformity in the lesions in contrast with the gross alterations. It is primarily a chronic proliferative process characterized by the development of a tough connective tissue sac distended with an abundant exudate so that an abscess-like structure is formed. There is little difference in the tissues between clinical cases and experimentally produced cases, except in age and extent of the process and the degree of necrosis.

The inner wall of the sac is the active layer of the inflammatory process. Some of that area is red and vascular and is the source of the abundant fibrinous exudate. Young granulation tissue forms under the fibrino-necrotic material with its pyknotic leucocytes. The cellular reaction in the wall of the bursal sacs is essentially one of chronic inflammation and consists of lymphocytes, plasma cells, fibroblasts, histiocytes, and an occasional giant cell. The rosettes, sometimes considered as pathognomonic for Actinomyces infection, are not encountered for they are associated only with "rough" strains of the infection. All of the 65 strains of *Actinomyces bovis* isolated from the cases of fistulous withers and poll evil in these studies were biochemically identical and of the "smooth" type. All of the rough strains studied in this laboratory so far have been non-proteolytic, while all smooth strains encountered were found to be definitely proteolytic. Herein may lie some of the confusion in evaluating bacteriologic studies on *A. bovis*. While there may be extensive necrosis in the ligament and much fibrino-necrotic material within the fistulous sacs, the inflammatory process is not one of true suppuration in the unopened lesions, for polymorphonuclear cells are uncommon.

ETIOLOGY

Three theories seem to have been suggested to explain the etiology of poll evil and fistulous withers: (1) the principle of mechanical injury, (2) infection of the ligamentum nuchae with *Onchocerca cervicalis*, and (3) bacterial infection.

The theory of mechanical injury as the exciting cause of fistulous withers has had the support of competent surgeons,[8,9,10,11] while others [1,5] have doubted that explanation. Freedom from mechanical injury would scarcely have explained the rarity of the condition in city horses, nor would it have accounted for its unusual prevalence in farm horses in some communities and but few cases in other areas.

Fistulous withers has been attributed[12,13,14] to an infection of the ligamentum nuchae with *O. cervicalis.* This theory seems scarcely adequate in the absence of further definite positive experimental studies. Furthermore, it is rare to find a normal ligament in old horses used for anatomic dissection and surgical exercises, for rarely are the ligaments of such horses free from necrosis and calcification at the withers and a high incidence of parasitic infection, even in the absence of fistulas.

The possibility that fistulous withers and poll evil might be infectious in nature has been suggested from time to time, but little in the way of concrete findings has been secured to support that theory until brucellar infection was incriminated. From all of those observations,[15,7,16,17,18] it has been shown that approximately 80 per cent of the affected horses show a positive blood agglutination test for *Br. abortus* and that, in a large proportion of the cases, the organism can be isolated from the fluid from the unopened cases.

This generalization likewise applies to our work. The fact that in a series of 107 cases of fistulous withers and poll evil treated in the college clinic, 98 of them reacted to the agglutination test at 1:25 and higher seems especially significant. On the other hand, among 64 horses with no trace of fistulous withers only 5 showed a reaction that approached a positive.

The isolation of *A. bovis* from cases of fistulous withers and poll evil of horses was reported by Kimball and Frank.[19] In the course of further work to date, this organism has been recovered from 65 out of 80 cases of fistulous withers and poll evil. With refinements in technique, the organism is regularly found in the hyperemic areas of actively proliferating granulation tissue on the inside wall of the affected bursal sacs.

The general conclusion from these bacteriologic studies on horses coming to the college clinic and those studied experimentally is that this process is not the result of a simple infection with *Br. abortus* or *Brucella suis* or with *A. bovis* alone, but rather results from a double or a complex infection with the two organisms. Those horses which have yielded *A. bovis* have likewise been examined for brucellosis by serologic, and in part by cultural, methods, and both infections have been found to be closely associated with the same lesions in practically all of the cases. This principle of a complex infection may seem somewhat unorthodox; yet it has several parallel examples, and, moreover, it is quite consistent with the association of horses with cattle. Horses with recently opened fistulous withers may be a source of infection for cattle and further serve to disseminate brucellosis and actinomycosis, although there is a question as to how long these infections may persist in the drainage from open cases. These horses should, however, be segregated from cattle.

The experimental studies with *Br. abortus, Br. suis* and with *A. Bovis* revealed that they are quite mildly invasive for the horse when infection is attempted with one of the organisms alone. A number of injections with *Br. abortus* and *Br. suis* were made into the supraspinous bursa of 3 experimental horses. Twelve experimental horses were inoculated in various ways with cultures of *A. bovis* which had been isolated from horses. These inoculations with the three respective organisms individually all resulted negatively. The combined cultures of *A. bovis* and *Br. abortus* or *Br. suis* were injected into the supraspinous bursa of 6 horses and 1 mule. In 1 horse, the injection procedure failed to induce any significant disturbance. In another, the bursitis apparently made a spontaneous recovery. In 4 horses and 1 mule, characteristic fistulous withers developed from the injection of the combined cultures. The experimental process appeared to be identical pathologically with those encountered under field conditions. Cultures of *A. bovis, Br. abortus* or *Br. suis,* respectively, were regularly recovered from these experimental fistulas, and the horses developed a strongly positive agglutination reaction for brucellosis; therefore, the dictum of Koch's postulates was fulfilled. Admittedly, the experimental inoculation of the combined cultures into the supraspinous bursa by injection may

not be equivalent to natural infection. The channel by which the Brucella and Actinomyces infections reach the respective bursae is obscure. We have no theory as to how or why these two bacterial infections localize in the supraspinous and supraatloid bursae. Perhaps the organisms find a favorable environment, or it may be a *locus minoris resistantiae.*

TREATMENT

The treatment of fistula of the withers or supraspinous bursitis should be determined by the changes that have occurred in the affected structures. Probably in no other condition affecting animals do we have more varied treatments and operations than for fistula of the withers. Probably in no other condition, likewise, do so many changes occur in the tissues if the case goes untreated for a long period of time. Due to the wide variety of changes that may occur in the tissues, as the result of the infection, the treatment will vary with the individual case.

The infection in fistula of the withers involves first the supraspinous bursa, and primary consideration should be given to this structure. The use of abortion vaccine has been recommended in the treatment, but we have not observed any beneficial results from its use. Due to the variation in size and position of the bursa, the symptoms will vary with the individual case. The bursa may slip laterally from its position beneath the ligamentum nuchae and pouch upward beneath the trapezius muscle. When this occurs, the bursa may be dissected out of the tissues with excellent results. If the bursa is surrounded and fastened down by the overlying tissue so it cannot become distended, it may rupture before any swelling is observed. When this occurs, there will be one or more openings discharging to the outside. When the bursa first ruptures, the discharge is serous in character, but in a short time it becomes purulent due to the secondary infection that gains access to the bursa. For these cases, it is indicated that complete drainage be established and the infection in the bursa be treated.

Other types of cases observed are those in which the bursa does not rupture but becomes gradually more extensive, due to the dilatations and distensions of the bursa as pockets are forced between the tissue layers. These various pockets or compartments are usually observed in the advanced cases. The openings between the various sacs may be very small, and the bilateral sacs may communicate under the ligamentum nuchae. In the treatment of these cases it may be very difficult to establish complete drainage from these numerous sacs or compartments. The infection in the bursa may invade the ligamentum nuchae or the spines of the vertebrae, which produces necrosis of these structures. When this occurs, an important part of the treatment consists in the removal of the necrotic portions of these structures.

CONCLUSIONS

This study was a fundamental inquiry into the anatomy, pathology, and etiology of equine poll evil and fistulous withers. The two processes are pathologically alike. It appears from the dissection of clinical and experimental cases that the process is primarily an inflammation of the supraatloid and supraspinous bursa, respectively. The pathology comprises a profuse serofibrinous exudate combined with varying amounts of necrosis, while the proliferative process associated with the exudation results in the formation of distended fistulous sacs of varying size and location.

Actinomyces bovis and *Brucella abortus* and *Brucella suis* have been regularly isolated from the lesions of a large number of cases of fistulous withers and poll evil. The injection of the combined cultures into the supraspinous bursa of experimental horses has produced a bursitis apparently identical with that of field cases. These significant bacteriologic findings are in agreement with many clinical observations in the field and indicate that horses acquire these infections through association with infected cattle. Infected horses may disseminate these infections to cattle.

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Hotis Test Reactions Produced by Toxicogenic, Coagulase-Positive Staphylococci

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SINCE 1943, the Hotis test has been included among the procedures employed in our investigations on bovine mastitis. It was a valuable aid for the detection of *Streptococcus agalactiae* infections, especially when employed in conjunction with other methods.[1]

Streptococcus agalactiae infections were reduced to a low level or were completely eradicated from several dairies through regular herd tests, followed by the segregation of infected cows during milking and the treatment of involved quarters with penicillin or other therapeutic agents.[2, 3, 4] Because of this accomplishment, it was possible to make a study of uncomplicated staphylococcic infections of the bovine udder and to make a critical analysis of the reactions produced in the Hotis test by staphylococci. It is the purpose of this paper to record the observations made on milk samples drawn at frequent intervals over a period of seven months from a self-contained dairy herd.

METHODS

Herd Management.—The herd consisted of 247 Holstein-Friesian cows, the majority of which were milked by machines and stripped by hand. About one-fourth of the herd was milked three times a day, the remainder twice daily. A sequence of milking was employed which was designed to reduce the spread of mammary gland infections. In addition, after each milking the teats were thoroughly moistened with a solution containing available chlorine (about 250 p.p.m.). A most important managerial factor affecting the present study was the exemplary preparation of the mammary glands prior to drawing the samples for bacteriologic study. As a result, significant bacterial contamination of the milk samples was rare.

The Hotis Test.—In 1936, Hotis and Miller[5] described a new method for the detection of *Str. agalactiae* in milk. It consisted of adding 0.5 cc. of a sterile 0.5 per cent aqueous bromcresol purple solution to 9.5 cc. of milk previously measured into a sterile test tube. The sample was incubated twenty-four to forty-eight hours at 37 C. and was observed for specific changes produced by bacterial growth.

The Hotis test was modified for use in the present study by drawing the milk directly into vials containing the bromcresol purple. Screw-capped, flat-bottomed vials measuring 18 by 110 mm. were employed. One cubic centimeter of a 0.33 per cent aqueous bromcresol purple solution was placed in each vial and was sterilized with steam at 15 lb. for thirty minutes. The bottom of a ¾-in. label when placed as high on the vial as possible indicated the level to which milk was to be drawn to provide a sample consisting of between 15 and 18 cc.

Changes in the Hotis samples after incubation were studied critically under a daylight fluorescent lamp. First the entire sidewall was examined for colonies or deposits, then the vial was gently inverted two or three times, and finally any deposit remaining on the bottom was carefully studied. A hand lens providing an enlargement of eight or ten times or a dissecting microscope fitted with an 8x ocular and a 1x objective was found to be helpful in ascertaining the structure of the deposits.

Microscopic Studies.—Smears were prepared from Hotis samples, which had been incubated for sixteen to twenty hours and subsequently shaken, and were stained by the Broadhurst-Paley method.[7] Microscopic findings were recorded according to a previously described code.[1]

Culture Technique.—Glenny and Stevens[8] demonstrated that pathogenic staphylococci produce either an *alpha* or *beta* toxin, or both toxins. Minett[9] has shown that defibrinated sheep or cow blood added to nutrient agar in Petri dishes provides a satisfactory medium for the demonstration of these toxins. The *alpha* toxin produces a zone of complete hemolysis around the colony, whereas the *beta* toxin produces a zone of darkened red cells. The majority of strains of pathogenic staphylococci from animal sources elaborate both toxins in varying proportions. For this reason, the pattern of the zone or zones is variable.

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In the studies reported in this paper, young dairy cows free from known infections with staphylococci were used as a source of blood for the culture medium.

In a preliminary trial, 412 routine Hotis samples were cultured in blood agar both before and after incubation. Each unincubated Hotis sample was diluted 1 to 10 in sterile saline and 1.0 cc. of this dilution was plated in veal infusion agar containing about 7 per cent fresh cow blood, whereas after the Hotis sample had been incubated for sixteen to twenty hours, a wire loopful was streaked on the surface of a veal infusion agar plate containing cow blood. Toxicogenic staphylococci, which also proved to be coagulase-positive, were isolated from 88 of the 412 plates prepared from the unincubated Hotis samples, whereas similar organisms were found on 117 plates streaked with the same milk after incubation. Because of this contrast between the two methods, the culturing of the milk after incubation was selected as the procedure for use throughout this study.

TABLE I—Correlation between Toxin-Producing and Plasma-Coagulating Abilities among 844 Staphylococcus Cultures Isolated from Different Bovine Milk Samples

Coagulase	No. cultures and types of reactions in agar containing cow blood			
	Typical toxin zones	Weak hemolysis	Slight hemolysis	No change
Pos.	542*	35*	11*	7
Neg.	2*	13	71	163

*Classified as pathogenic staphylococci—total 590.

The Coagulase Test.—Chapman[10] and others have shown that, in addition to the production of toxins, another characteristic of pathogenic staphylococci is their ability to coagulate rabbit or human plasma. The method suggested by Chapman,[11] with slight modifications, was used. The cultures were established in broth from single colonies, were transferred to veal infusion agar slants, and were incubated overnight at 37 C. A 4-mm. loopful of each culture was suspended in 0.6 cc. of 0.4 per cent saline to which was added 0.2 cc. of fresh rabbit plasma. The tests were conducted in small serologic tubes measuring ⅜ by 3 in. The tubes were placed in a water bath at 37 C. for four hours, examined for clot formation, kept overnight at room temperature, and examined again.

RESULTS

During the period of seven months, either composite or quarter samples, totaling 2,889, were drawn for study. A representative colony was transferred to broth from each blood agar plate containing staphylococci showing typical toxin zones or narrow hemolytic zones. The latter were classified as weakly and slightly hemolytic staphylococci, and they were regarded as toxicogenic strains only when they also coagulated rabbit plasma. A considerable number of milk samples contained staphylococci which did not produce visible changes in cow blood agar. These were called inert types and at first a culture was isolated from each plate showing such colonies, but since the inert staphylococci almost invariably proved to be coagulase-negative, only occasional isolations were made thereafter.

The data in table 1 show the correlation between the toxin-producing and the plasma-coagulating abilities among the 844 strains of staphylococci isolated from different milk samples. A culture was classified as pathogenic when it manifested both the ability to produce toxins and the ability to coagulate rabbit plasma. An exception to this rule was made with two cultures, which produced typical toxin reactions on cow blood agar but failed to coagulate rabbit plasma. This decision was made because toxicogenic, coagulase-positive staphylococci were isolated from milk samples taken from the same mammary quarters on previous and subsequent samplings. Pathogenic staphylococci were demonstrated in 590 of the 2,889 milk samples.

Cultures which were weakly or slightly hemolytic but which failed to coagulate rabbit plasma were classified as nonpathogenic. Likewise, all inert types were regarded as nonpathogenic, even though seven cultures among the 170 tested did coagulate rabbit plasma. Five of these seven cultures came from mammary quarters which on previous and/or subsequent samplings failed to show coagulase-positive strains of either inert or toxicogenic staphylococci, whereas two of the cultures were isolated from the same mammary quarter on separate samplings.

The specific names *Staphylococcus aureus* and *Staphylococcus albus* are not used in this paper because a critical study of the chromogenesis of the cultures was not made.

The Microscopic Examination.—Among

the 2,889 milk smears, staphylococci were seen on microscopic study in 749. Of the 749 microscopically positive smears, 419 (55.9%) were prepared from milk samples subsequently shown to contain pathogenic staphylococci, whereas 330 smears (44.1%) were prepared from milk samples containing inert types of staphylococci. An abnormal number of leucocytes was observed in only 12.4 per cent of the 419 smears. As previously stated, pathogenic staphylococci were found in 590 of the 2,889 milk samples by streaking the milk after incubation on blood agar plates, whereas the microscopic study detected staphylococci in 419 (71.0%) of the 590 samples. These data demonstrate that the microscopic examination of stained smears prepared from incubated milk is not a sufficiently accurate procedure to be used as a sole method of the selection of milk samples containing pathogenic staphylococci.

The Hotis Reaction after Sixteen to Twenty Hours' Incubation.—Murphy,[12] in an analysis of the Hotis test results covering an eleven-month study of 140 cows in a single herd, had 1,145 samples in which staphylococci were found. He did not attempt to classify these staphylococci as pathogenic or nonpathogenic strains. Murphy reports that among the Hotis samples containing staphylococci, 11.4 per cent showed small, white or pale yellow colonies with chocolate-colored centers or small, chocolate-colored colonies with white or pale yellow centers; 14.4 per cent revealed small, white colonies in a blue column of milk; and, 14.1 per cent showed a slight granular sediment, white or pale yellow in color. Miller,[6] in his analysis of over 10,000 Hotis samples states, "Rust-colored colonies are always indicative of *Staphylococcus aureus.* There is a great deal of variation in the ability of different strains of this Staphylococcus to ferment lactose. When strains producing more acid are present, the milk assumes a more definite yellow shade and the flakes are more yellow in color. The center of the flake is yellow or white and the wide outer border is rust-colored. At times only one or two small flakes may be present, and a careful examination of the bottom of the tube will be required to find them. All milk samples containing staphylococci do not show the characteristic rust-colored flakes, but when they are present they are definitely diagnostic of this infection."

In the present study, eight closely related Hotis reactions were selected as indi-

TABLE 2—Closely Related Reactions in the Hotis Test, After Sixteen to Twenty Hours of Incubation, Correlated with the Presence of Pathogenic or Nonpathogenic Staphylococci in the Milk

	Description of Hotis reaction	No. out of 2,889 Hotis tests showing reaction and containing: Pathogenic staphylococci	Nonpathogenic staphylococci or other organisms
1)	Green colonies, with white centers, adhering to the bottom of vial	154	26
2)	Same as 1, with similar colonies on sidewall	12	0
3)	Brownish green colonies, with white centers, adhering to bottom of vial	56	1
4)	Same as 3, with similar colonies on sidewall	30	1
5)	Brown or russet colonies, with white centers, adhering to bottom (plate 1)	40	2
6)	Same as 5, with similar colonies on sidewall	10	2
7)	Adherent brown central sediment with a peripheral ring or crescent of whey held at the bottom by a variable degree of coagulation of the milk above	19	0
8)	Complete digestion producing a greenish or yellow translucent whey with a brown or green residue usually extending up one side of the vial in the form of a cylindrical mass or a flocculent deposit (plate 3)	5	0
	Totals	326 +	32 = 358
	Percentage of reliability	91.1 +	8.9 = 100%

cative of pathogenic staphylococci, and these reactions are described in table 2. It is shown in this table that, among the 2,889 Hotis samples, the selected reactions occurred 358 times, and of these, 326 (91.1%) were in samples containing toxicogenic, coagulase-positive staphylococci, whereas 32 (8.9%) were in Hotis samples revealing either nonpathogenic staphylococci or other organisms. The total number of milk samples containing pathogenic staphylococci was 590, but 31 of these also contained an abundance of streptococci or coliform organisms which dominated the Hotis reactions and, therefore, masked the changes which may have been produced by the staphylococci. These 31 samples were removed from the data leaving 559 samples for comparative study. It is seen then that, after sixteen to twenty hours at 37C., 326 (58.3%) of the 559 samples containing pathogenic staphylococci manifested reactions in the Hotis test which were regarded as typical of toxicogenic, coagulase-positive staphylococci.

In addition to the changes listed in table 2, adherent gray colonies with white centers were observed in 81 of the Hotis samples. Thirty-seven of these samples (45.7%) contained toxicogenic, coagulase-positive staphylococci, whereas the remainder revealed either nonpathogenic strains of staphylococci or other organisms. Therefore, the occurrence of gray colonies in Hotis samples after sixteen to twenty hours of incubation was not considered highly indicative of pathogenic staphylococci.

The Hotis Reaction after Forty Hours' Incubation.—During the latter half of this investigation, the Hotis samples were returned to the incubator and examined again after a total incubation period of forty hours. Certain samples which were negative on the first reading developed typical colonies with white centers during the second period of incubation. Many of the samples showing this delayed colony development contained toxicogenic, coagulase-positive staphylococci, but a significant number of such samples revealed only nonpathogenic strains. For this reason, the formation of typical colonies during the second period of incubation was not considered to be a reliable indication of the presence of pathogenic staphylococci. However, it is felt that this phase of the problem should receive further study.

The second period of incubation led to a characteristic digestion of many Hotis samples containing toxicogenic, coagulase-positive staphylococci. The extent of digestion at the end of forty hours of incubation varied from sample to sample (plate 2). The initial change consisted of the formation of a brown or green adherent sediment associated with a crescent of clear whey in the angle formed by juncture of the bottom and sidewall of the vial. This crescent of clear fluid was held in place by a partial to complete coagulation of the milk above. The next stage in the process of digestion consisted of an expansion of the digested area to form a clear peripheral ring about a central, brown or green sediment. Upon inverting the vial, it was usually observed that the central sediment was continuous above with a grayish or greenish cylindrical column or plug of coagulated milk. The

TABLE 3—Comparison, on 1,733 Hotis Samples, of the Relative Efficiency of the First and the Second Readings for the Detection of Pathogenic Staphylococci

No. Staph.-pos. milk samples	Incubation time	Hotis samples showing typical reactions					% Staph.-pos. samples detected by Hotis test
		Total no.	True-pos.* No.	%	False-pos.† No.	%	
325	20 hr. (1st reading)	231	216	93.5	15	6.5	66.6
	40 hr. (2nd reading)	235	227	96.5	8	3.5	69.8
	Results (1st & 2nd readings combined)	281	261‡	92.9	20§	7.1	80.0

*Pathogenic staphylococci isolated on blood agar. †No pathogenic staphylococci found on blood agar. ‡190 samples were positive on both readings; 26 samples were positive on the first reading but did not become digested during the second period of incubation; and 45 samples were positive on the second reading only. §Three samples gave false-positive reaction on both the first and second readings.

final stage in the process consisted of a more or less complete digestion of the entire sample, characterized by a yellow or greenish translucent whey associated with a brown, brownish green, or green residue (plate 3). The residue usually appeared in the form of a cylindrical plug adhering to the bottom of the vial and extending upward along one side of the vial. Occasionally, the pluglike mass terminated a short distance above the bottom of the vial and a variable quantity of flocculent, brown, brownish green, or green residue was found on one side of the vial. Infrequently, the flocculent residue was scattered over more than just one side of the vial. In a few instances, the typical residue was not in the form of a pluglike mass beginning at the bottom of the vial, but instead it was found loose in the vial or floating at the top of the sample.

Digestion of the Hotis sample was also produced by certain other species of organisms. Infrequently, certain large rods, similar to *Bacillus subtilis*, which were assumed to be contaminants, were found to produce a digestion of the samples which was characterized by a more or less purplish whey associated with a bluish gray residue. Coliform organisms also produced digestion of the milk on prolonged incubation. With such samples, both the whey and the residue were yellow and contained gas.

Comparison of Hotis Reactions after Twenty and Forty Hours' Incubation.—For the purpose of this comparison the sixteen- to twenty-hour incubation period will be referred to as the first reading and the forty-hour incubation will be designated as the second reading. A summary is presented in table 3.

First and second readings were made on 1,733 Hotis samples among which 360 contained pathogenic staphylococci. Only 325 of these 360 samples were suitable for the comparative study since the remainder also contained streptococci or coliform organisms which dominated the Hotis reactions, especially during the second period of incubation. Changes indicative of pathogenic staphylococci were exhibited by 231 of the samples on the first reading, of which 216 samples (93.5%) contained toxicogenic, coagulase-positive staphylococci, whereas 15 samples (6.5%) revealed either nonpathogenic strains of staphylococci or other organisms. The 216 true-positive reactions represent 66.6 per cent of the samples containing pathogenic staphylococci.

At the second reading, 235 Hotis samples were in various stages of the characteristic digestion. Of this group, 227 samples (96.5%) contained toxicogenic, coagulase-positive staphylococci, whereas only eight samples (3.5%) did not contain pathogenic staphylococci. This group of 227 true-positive reactions represents 69.8 per cent of the samples in which pathogenic staphylococci were demonstrated.

A comparison of the results of both readings on individual samples containing pathogenic staphylococci reveals that 190 were

TABLE 4—Relation of the Number of Toxicogenic, Coagulase-Positive Staphylococci Inoculated into Sterile Hotis Samples to the Changes Produced During Twenty Hours of Incubation at 37 C.

Dilution of broth culture	No. Staph. colonies developing in blood agar from the inoculum (0.1 cc.)	Hotis reaction after 20 hr. incubation at 37 C.
Undiluted	*	Complete digestion. Brown residue in the form of a cylindrical column adhering to sidewall.
10^{-3}	*	Brown central sediment surrounded by a ring of whey; green residue and colonies adhering to sidewall.
10^{-4}	*	Brown sediment with a crescent of whey in the angle formed by bottom and sidewall of vial; greenish brown colonies on sidewall.
10^{-6}	*	Brown sediment associated with beginning crescent of whey; few greenish brown colonies on sidewall.
10^{-8}	*	Greenish brown sediment covering bottom and showing many white colony centers.
10^{-10}	1,040 toxicogenic staph. colonies	Many green colonies with white centers, adhering to bottom of vial.

* = Too high to count.

positive on both readings; 45 were negative on the first reading but manifested the characteristic digestion after the second period of incubation; and 26 were positive on the first reading but failed to become digested during the second period of incubation. The last observation indicates that not all strains of toxicogenic, coagulase-positive staphylococci that produce reactions during the first period of incubation proceed to digest the milk during the second period of incubation. This suggests that both a first and second reading should be made in order to detect a maximum number of samples containing pathogenic staphylococci. A combination of the two readings in this trial led to the detection of 261 (80.0%) of the 325 samples containing pathogenic staphylococci, whereas 20 samples gave false-positive reactions, representing an error of 7.1 per cent.

EXPERIMENTAL

In order to be more certain that the Hotis reactions observed in the field trials were actually produced by pathogenic strains of staphylococci, sterile raw milk was obtained, was distributed aseptically into sterile Hotis vials, and was inoculated with pure cultures of either pathogenic or nonpathogenic strains of staphylococci. McCulloch and Fuller[13] had shown that aseptically drawn milk, obtained from quarters consistently negative to the Hotis reaction, supported typical Hotis reactions when inoculated with a known strain of *Str. agalactiae*. Heating the milk to 80 C. for five minutes destroyed the ability of the milk to support the Hotis reaction typical of *Str. agalactiae*, but the addition of equine or bovine serum reactivated the milk or supplied that which had been destroyed.

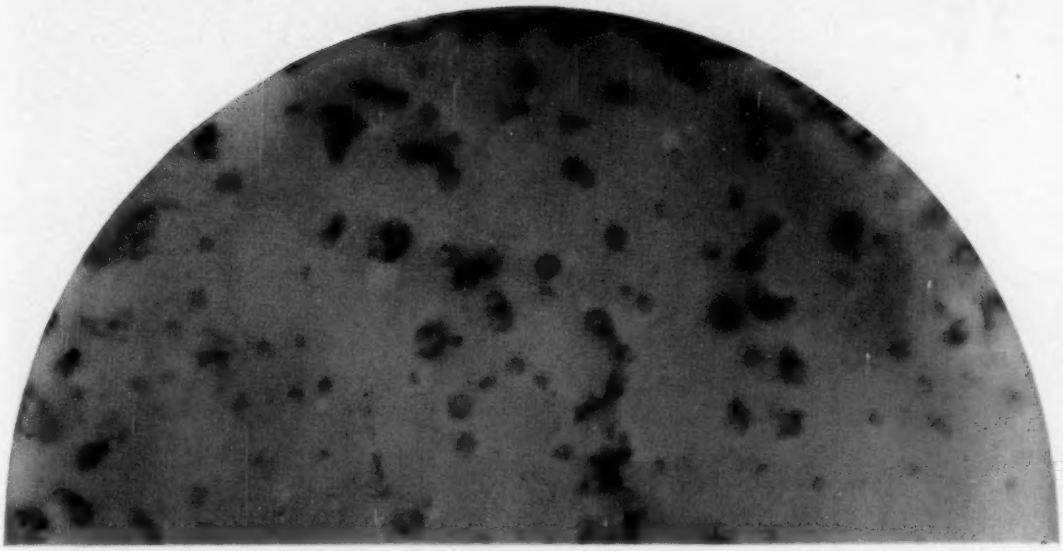
A young animal was selected that had consistently given sterile milk on previous samplings. The milk was drawn as follows: The udder was thoroughly washed with running water and then dried with a clean towel. This was followed by a second washing with a clean cloth saturated with chlorine solution and finally the teat orifice of the quarter selected was treated with isopropyl alcohol, and immediately thereafter the milk was allowed to flow by gravity into a sterile liter bottle *via* a sterile rubber hose and teat cannula. The aseptically drawn milk was refrigerated until it was distributed into sterile Hotis vials containing the bromcresol purple solution.

The aseptically prepared Hotis samples were tested for sterility by incubating them at 37 C. for twenty hours followed by streaking a wire loopful of each sample on veal infusion agar containing cow blood. When the test for sterility indicated contamination, the sample was discarded. However, occasionally a few tiny colonies of a small bacillus developed on the sterility test plate, but since it was determined that this organism did not grow profusely in the milk or alter the Hotis sample, milk containing it was not removed from the trials.

Effect of the Number of Staphylococci in the Inoculum on the Hotis Sample.—In the study of the reactions induced by pure cultures of either pathogenic or nonpathogenic staphylococci in Hotis samples prepared from sterile milk, it was necessary to determine the size of the inoculum to be used. This in turn led to an interesting observation on the relationship of the number of staphylococci in the milk, prior to incubation, to the extent of change in the sample during incubation.

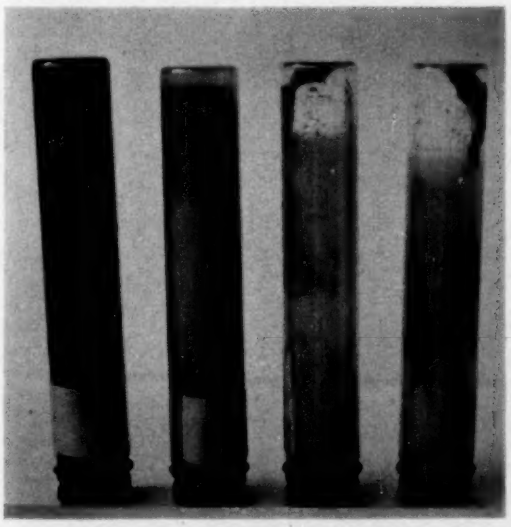
A pure culture of a toxicogenic, coagulase-positive Staphylococcus obtained from a field Hotis sample, that had shown typical colonies on the first reading and characteristic digestion on the second reading, was selected for this trial. The culture was grown in serum broth for twenty hours and then serial dilutions were prepared from it in sterile physiologic saline solution. A 0.1-cc. inoculum of the undiluted broth culture was introduced into a Hotis vial containing sterile milk, and other Hotis vials were inoculated with the saline dilutions. Similar quantities of culture and each dilution of it were plated in veal infusion agar containing cow blood in order to ascertain the relative number of staphylococci introduced into each Hotis vial. The Hotis samples and agar plates were incubated at 37 C. for twenty hours. The results of this trial are presented in table 4.

The actual number of colonies developing in the blood agar plates was too large to permit a count in all but the plate prepared from the highest dilution. It is obvious, however, that the number of staphylococcic cells introduced into the Hotis samples decreased as the dilution of the original culture was increased. The reactions in the Hotis samples presented a graded series of changes which lessened in intensity in direct proportion to the decrease in number of organisms in the inoculums. These changes ranged from complete digestion to isolated green colonies, with white centers, on the bottom of the vial. These observations suggest that, with routine field samples containing toxicogenic, coagulase-positive staphylococci which are capable of digesting the milk, the degree of change during twenty hours of incubation is a suggestive index of the rela-



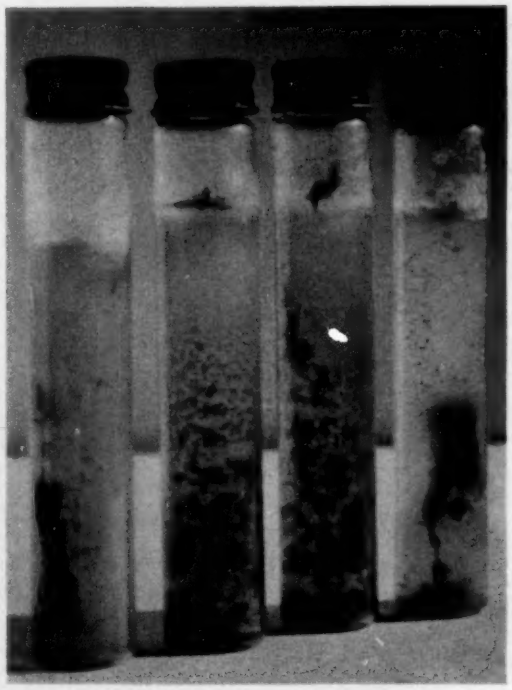
—*A. E. Graffius.*

Fig. 1—View of one half of the bottom of a Hotis test vial, after twenty hours at 37 C., showing adherent brown colonies with white centers. This reaction was produced by pathogenic staphylococci.



—*A. E. Graffius.*

Fig. 2—Four Hotis test samples containing pathogenic staphylococci. The samples were incubated at 37 C. for forty hours and they show the various stages (l. to r.) of a characteristic digestion caused by pathogenic staphylococci. The vials have been inverted to display the reactions more advantageously.



—*A. E. Graffius.*

Fig. 3—Four Hotis test samples showing the complete digestion produced by pathogenic staphylococci during forty hours of incubation at 37 C. The milk has been changed to a yellowish translucent whey and a brownish residue. The residue is in the form of a cylindrical plug in the vials on each end, whereas in the two center vials it appears as a flocculent deposit on one sidewall.

tive number of staphylococci in the milk prior to incubation.

Hotis Reactions Produced by Pure Cultures of Staphylococci Selected at Random.—Aseptically prepared Hotis samples were inoculated with 0.1 cc. of young broth cultures of staphylococci obtained by single colony isolations from the blood agar plates which had been streaked with the routine field samples of milk. No attempt was made to select cultures on the basis of positive Hotis reactions in the field samples, but instead a random sampling of both toxicogenic and inert staphylococci appearing on the blood agar plates was made. One Hotis vial was left as an uninoculated control for each ten vials inoculated. In addition, after the vials had been incubated for twenty hours, each sample was streaked on veal infusion agar containing cow blood to demonstrate the presence or absence of growth. The results of these trials are summarized in table 5.

During the first part of this study the incubation period was limited to twenty hours. The samples in this phase of the experiment are designated as series 1. Thirty-seven pathogenic and 44 nonpathogenic strains of staphylococci were employed. It was observed that 83.7 per cent of the toxicogenic, coagulase-positive cultures produced Hotis reactions similar to those listed in table 2, while only 4.5 per cent of the nonpathogenic strains reacted in this manner.

In the field samples of milk containing toxicogenic, coagulase-positive staphylococci are highly specific for pathogenic strains of staphylococci and have diagnostic value. The fact that between 15 and 20 per cent of the strains of pathogenic staphylococci, in pure culture, failed to produce typical reactions in Hotis samples prepared from sterile milk leads one to the supposition that not all strains of toxicogenic, coagulase-positive staphylococci have the ability to actively elaborate the specific enzymes necessary for the digestion of milk.

A preliminary trial was made to determine whether the ability to digest milk or failure to do so was constant with a given strain. For this purpose, Staphylococcus cultures were reisolated from ten Hotis samples in series 2. Four of these samples had shown the characteristic digestion, whereas six had not. Hotis samples prepared from sterile milk were again inoculated with 0.1 cc. of young broth cultures of the reisolated strains. The inoculated milk was then incubated at 37 C. for one week. The four cultures which had produced digestion in the first trial during forty hours of incubation did so again in this second trial. One of the six cultures which had not digested the milk in the first trial did produce digestion between the fortieth and sixtieth hours of incubation in the second trial, and the remaining five cultures produced digestion on the sixth day of incubation.

TABLE 5—Reactions Observed in Hotis Samples Prepared from Sterile Milk, Inoculated with 0.1 cc. of Twenty-Hour Broth Cultures of Either Pathogenic or Nonpathogenic Strains of Staphylococci

Series	No. staph. cultures	Type culture	Hotis reaction after 20 hr. incubation: Type reactions and no. cultures producing them* 2	3	4	6	7	8	Total pos. reac.	% pos. reac.	Hotis reaction after 40 hr. incubation: Type reactions and no. cultures producing them* 7	8	Total pos. reac.	% pos. reac.
1†	37	Path.	0	1	1	1	20	8	31	83.7	..	..	..	..
	44	Non path.	0	2	0	0	0	0	2	4.5	..	..	..	..
2	55	Path.	1	0	0	0	35	4	40	72.7	2	43	45	81.8
	10	Non path.	0	0	0	0	0	0	0	00.0	0	0	0	00.0

*The numbers used for the type reactions correspond to the numbers employed in describing the reactions listed in table 2. †The Hotis samples in series 1 were incubated for twenty hours only.

In series 2, 55 pathogenic and ten nonpathogenic strains were employed, and readings were made after both twenty and forty hours of incubation. In these trials, 72.7 per cent of the pathogenic staphylococci caused positive reactions in the first twenty hours of incubation, and after forty hours 81.8 per cent of the samples were positive. The ten nonpathogenic strains did not induce typical reactions.

These data demonstrate the validity of the assumption that the Hotis reactions observed

DISCUSSION

The data presented in this paper are the results of an investigation conducted on milk samples obtained from a single dairy herd. No attempt has been made to pursue equally critical studies in other dairy herds. No conclusions are being drawn at this time relative to the applicability of these results to dairy herds in general, and it is not the intent of this paper to suggest that the

Hotis test may be used to supplant methods now in use for the demonstration of pathogenic staphylococci in milk samples. It is the writer's purpose, however, to point out that certain Hotis reactions were found to be indicative of pathogenic staphylococci, with the hope that others will seek to determine whether or not similar results can be obtained in controlled investigations in herds in other sections of the country.

It was learned from this study that when mammary quarters harbor both pathogenic staphylococci and *Str. agalactiae* or coliform organisms, the last two species if present in abundance will rapidly ferment the milk sugar, and this in turn will mask or prevent the development of the typical reactions due to pathogenic staphylococci. On the other hand, if in such mixed infections the pathogenic staphylococci predominate, they may produce their specific reactions, but the colonies, sediment, or residue may be tinged with yellow as a result of the acid produced from lactose by the concomitant infections.

It is reasonable to assume that species of bacteria not normally found in the udder may also produce reactions in Hotis samples during the incubation which may be indistinguishable from the changes induced by pathogenic staphylococci. For this reason it cannot be overemphasized that the udder and teats must be washed free of visible extraneous material followed by adequate disinfection of the teat orifices prior to the collection of milk samples for bacteriologic study.

SUMMARY

1) Results from 2,889 milk samples have been analyzed. The milk was subjected to the Hotis test, to microscopic studies of stained smears prepared from the incubated Hotis samples, and to the streaking of the incubated samples on veal infusion agar containing cow blood for the demonstration and isolation of toxicogenic staphylococci.

2) Pathogenic staphylococci (toxicogenic and coagulase-positive) were found in 590 samples.

3) Microscopic examination revealed staphylococci in 71.0 per cent of the samples shown to contain pathogenic staphylococci. In 12.4 per cent of these smears, a concomitant increase in leucocytes was observed. However, staphylococci were also observed in smears prepared from milk not infected with pathogenic staphylococci. Forty-four per cent of the total smears revealing staphylococci fell in the latter category.

4) After sixteen to twenty hours of incubation the principal Hotis reactions indicative of pathogenic staphylococci were the occurrence of adherent green, greenish brown, brown, or russet-colored colonies with white centers. By attributing diagnostic significance to these reactions, 58.3 per cent of samples containing pathogenic staphylococci were detected, whereas an 8.9 per cent error was encountered due to false-positive reactions produced by nonpathogenic staphylococci or other organisms.

5) Upon incubation of the Hotis samples for forty hours, it was found that a characteristic digestion was indicative of the presence of pathogenic staphylococci. A few samples showing typical colonies after twenty hours of incubation failed to digest the sample during the second period of incubation. Thus, it was necessary to observe the Hotis samples both after twenty hours and forty hours of incubation in order to detect the maximum number containing pathogenic staphylococci. A combination of the two readings made on 1,733 Hotis samples, among which 325 contained pathogenic staphylococci, led to the detection of 80.0 per cent of the 325 samples. However, 20 false-positive reactions were recorded, leading to a 7.1 per cent error.

6) Hotis samples were prepared from sterile raw milk and inoculated with 0.1 cc. of pure broth cultures of either pathogenic or nonpathogenic staphylococci. In one series consisting of 37 pathogenic and 44 nonpathogenic strains, 83.7 per cent of the former cultures produced the typical Hotis reactions, whereas only 4.5 per cent of the nonpathogenic strains produced positive reactions during twenty hours of incubation. In a second series consisting of 55 pathogenic and ten nonpathogenic Staphylococcus cultures, the inoculated Hotis sam-

ples were incubated for a total of forty hours. After twenty hours, 72.7 per cent of the pathogenic strains had produced positive Hotis reactions, and by the end of forty hours of incubation, 81.8 per cent of the pathogenic staphylococci had produced digestion of the milk. The ten nonpathogenic strains of staphylococci did not cause positive reactions.

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DISCUSSION

DR. L. P. DOYLE (Lafayette, Ind.).—I didn't get the relationship between the presence of these pathogenic staphylococci and definite evidence of mastitis.

DR. SCHALM.—The only evidence we had of mastitis was an increase in leucocytes. Twelve per cent of the smears from microscopic study in which staphylococci occurred showed an abnormal leucocyte count.

DR. J. W. WALKER (Hulmeville, Pa.).—I congratulate Dr. Schalm. We have run about 20,000 comparisons of the Hotis test at the University of Pennsylvania, and I think our work will correlate very closely. The pictures are very good.

How much dye do you use?

DR. SCHALM.—We use 0.33 per cent. It works out to the same concentration that Hotis originally used—0.5 cc. of a 0.5 per cent in 9.5 cc. of milk. We used 1.0 cc. of a 0.33 per cent for about 15 cc. of milk.

DR. WALKER.—Have you decreased the amount of dye experimentally?

DR. SCHALM.—No.

DR. WALKER.—We thought the dye might have an inhibiting action and are studying that.

DR. SCHALM.—If the dye is reduced too much, color reactions will be poor. The colonies are very pale; in fact, there would be only the white centers. There would be no periphery of green or brown.

DR. WALKER.—Did I understand you to say this would pick out 80 per cent

DR. SCHALM.—By combining the results of the first day's reading, in which we look for typical colonies, with the second day's reading, at which time we look for typical digestion we selected 80 per cent of the milk samples subsequently shown to contain pathogenic staphylococci.

We had approximately 7 per cent error, consisting of milk samples in which either inert or nonpathogenic staphylococci were present and which produced changes indistinguishable from those we have described.

DR. WALKER.—Would it be possible to have staphylococci and not get a positive result?

DR. SCHALM.—Yes, it is possible. I neglected to bring that out. It seems that about 20 per cent of staphylococci picked at random and placed in sterile milk fail to produce these changes. They produce other changes but not those I have described. There apparently are some strains of pathogenic staphylococci incapable of altering the Hotis test in the manner described in this paper.

DR. MARKMAN (Maryland).—I want to thank you for an interesting paper. Having been closely associated with the Hotis test for six or eight years, I fully appreciate this excellent paper.

However, have you noted any correlation between the number of staphylococci in the original sample and the degree of digestion in the Hotis test.

DR. SCHALM.—Our conclusion is that, if digestion occurs in the sixteen to twenty hours or during the first day's incubation, it is a suggestive index of a large number of organisms shed from the udder at the time the milk is drawn, whereas if only a few colonies appear at the bottom of the tube, it is an indication that the number shed is limited.

DR. MARKMAN.—That has been our observation. I was interested to know your results. Could not the longer incubation be responsible for the increased percentage of samples which showed digestion on the second day?

DR. SCHALM.—Yes. Prolonging the incubation period permits the samples containing very few organisms to build up a population of organisms which will digest the milk on the second day. I have prolonged the incubation for sixty and eighty hours, even for a week, but I was reluctant to report on that here. As the procedure is prolonged, other organisms begin to digest the milk and results are confusing. I would not like to see the practicing veterinarian adopt these findings immediately. I would rather that research workers who can conduct controlled experiments determine whether or not what I have said is valid.

DR. R. ALLEN PACKER (Ames, Iowa).—How often did spore-forming bacilli interfere with the reading of digestion? In spite of all the precautions taken, there is an occasional sample with spore-forming bacilli which are very rapid in their digestion of milk and which will give reactions similar to those described. How often did that interfere with the reading?

DR. SCHALM.—It will interfere to a great extent unless the samples are taken in a clean manner. Factors which assist are: the microscopic test and finding of the staphylococci in the digested samples. Digestion of Hotis samples by spore-forming bacilli can usually be distinguished from digestion caused by pathogenic staphylococci. The whey is usually alkaline or purplish in the case of the former organisms, and the residue assumes a gray or purple color.

MEMBER.—Will you comment on finding evidence of mastitis in only 12 per cent of the quarters from which you isolated apparently pathogenic staphylococci.

DR. SCHALM.—That is a difficult question to discuss. Environmental factors and management definitely influence the seriousness of mastitis in a dairy herd. This herd was well managed and had only 1 case of gangrenous mastitis in the last three years, while many herds studied, having similar organisms, have had tremendous trouble from gangrenous mastitis.

MEMBER.—That is the same conclusion we reached.

We have found that herds carrying a large percentage of cows infected with staphylococcus, as long as good milking practices are observed, apparently have little trouble; but when there is a break down in the milking practices, such as the milking machine going out of order or even changing milkers, almost immediately an epidemic of mastitis will occur.

The Experimental Alteration of Blood Potassium and Calcium Levels in Cattle

JOE DENNIS, Ph.D., and F. G. HARBAUGH, D.V.M.

Lubbock, Texas

IN THE PAPER[5] immediately prior to this one it has been shown that cattle suffering from symptoms of tetany as a presumed result of grazing wheat often have low serum calcium levels. The plasma potassium content of the blood of the majority of these animals has been either normal or lower than normal. In the case of 1 animal studied, the plasma potassium was high. This was the only animal studied which exhibited symptoms of extreme hypersensitiveness which are often described in connection with this condition. In all cases studied, the symptoms of tetany were observed when the potassium to calcium ratio exceeded 2.25. It seemed appropriate to determine whether the observed symptoms could be produced experimentally by manipulation of the blood level of these two elements. We have attempted to alter the ratio of these two elements by procedures designed to (1) raise the potassium level, (2) lower calcium, and (3) alter the value of both elements simultaneously.

EXPERIMENTAL

Samples of blood for analysis were drawn from the jugular vein in all cases. Potassium determinations were made on plasma. Sodium oxalate was used as an anticoagulant in these samples. The analytical method employed was essentially that of Breh and Gaebler,[1] followed by the diazotization procedure of Briggs[2]. The calcium determinations were run on serum using the Clark-Collip modification of the Kramer-Tisdall method.[3]

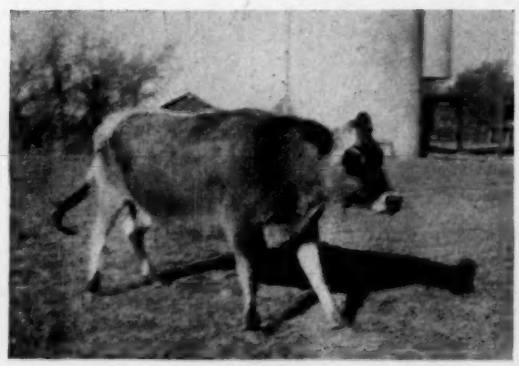

Fig. 1—Showing the first observable results of the administration of potassium chloride to a cow by stomach tube.

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1) *Experiments Involving the Feeding of KCl Alone.*—A Jersey cow weighing 680 lb. was placed on a diet of ground dairy feed and nonlegume hay. Potassium chloride (KCl) was added to the ground feed beginning with the addition of 81 Gm. each morning and night and increasing the amount until, at the end of six days, she was receiving 300 Gm. of the salt both morning and night. This daily amount of KCl was calculated to be equivalent to about one half of the amount of potassium which an average cow would consume per day when grazing on good wheat pasture. The feeding was continued for twenty days. At the end of this time, a total of about 9,200 Gm. of the salt had been consumed. Samples of blood were analyzed at intervals during the course of the experiment. The results of the analyses are shown in table 1. The animal appeared to be normal at all times.

2) *Experiments Involving the Feeding of KCl and $K_3C_6H_5O_7 \cdot H_2O$.*—A Jersey cow weighing 640 lb. received the same ration as the cow in experiment 1. Potassium chloride and potassium citrate ($K_3C_6H_5O_7 \cdot H_2O$) were mixed with her ground feed beginning with 50 Gm. of each salt both morning and night. The amounts were increased until, at the end of four days, she was receiving 150 Gm. of each at both the morning and night feeding. The experiment was continued for sixteen days. A total of approximately 4,000 Gm. of each salt was consumed during this time. The results of the analyses of the blood samples taken during the course of this experiment are given in table 2.

3) *Experiments Involving the Administration of KCl by Stomach Tube.*—(a) Experiment 1.—A Jersey cow weighing 640 lb. was placed in a stanchion. A stomach tube was passed and adjusted to such a position as deemed necessary to permit the administered KCl solution to spiral down the esophogeal groove and be deposited largely in the

abomasum. This was done by passing the stomach tube nearly to the cardia of the stomach and pumping the KCl solution in very slowly. The proper length of the tube to be passed was determined by external measurement, and the length of the tube inserted was not sufficient for it to enter the stomach. The amount of KCl administered was 648 Gm. dissolved in 2 gal. of water. Since this was the first experiment of this sort, particularly spectacular results were not anticipated, and no preliminary normal sample of blood was obtained; however, this cow had shown a normal blood potassium level of about 16 mg./100 cc. for several days prior to the experiment. About eight minutes after the KCl was administered, the animal trembled and went into tetanic convulsions with opisthotonus and limbs extended. When attempts were made to handle her, it brought about violent struggling which turned her completely on her back. The spasms resembled the death struggle of cows. With the aid of three men, the animal was held, a needle inserted into the left jungular vein, and a sample of blood collected for analysis. An injection of 425 cc. of calcium dextrose solution containing 10.5 Gm. of calcium was administered, and within five minutes of finishing the injection the animal was on her feet and eating hay. Ten minutes later another sample of blood was collected for analysis. The results of the analyses are shown in table 3. We believe that the rapid rise which was observed in the blood potassium level is most easily explained on the assumption that some of the administered KCl reached not only the abomasum but also the initial portion of the intestine.

TABLE 1—Showing the Effect of Feeding Potassium Chloride on the Potassium and Calcium Level of Cow's Blood

Total amt. KCl prior to sampling (Gm.)	No. days receiving KCl	Plasma K mg./100 cc.	Serum Ca mg./100 cc.	Ratio K:Ca
0	0	15.6	11.2	1.38
0	0	16.2	10.6	1.53
593	3	19.6	10.5	1.86
1393	5	17.4	10.7	1.62
4343	10	22.9	11.4	2.01
5543	12	25.0	10.7	2.33
8093	17	19.9	10.3	1.93
9293	20	19.6	10.9	1.80

(b) Experiment 2.—A Jersey cow weighing 680 lb. was placed in a stanchion and a sample of blood collected from the left jugular vein. The KCl, 400 Gm. dissolved in 2 gal. of water, was administered as described in experiment 1; an attempt was made to place it in the abomasum. The animal appeared quite normal in all respects during the course of the experiment. Twenty minutes later a second blood sample was collected for analysis. The results are shown in table 3.

(c) Experiment 3.—Two days later the animal used in experiment 2 was subjected to a similar experiment. The amount of KCl administered was increased to 648 Gm. In six and one-half minutes the gluteal muscles trembled, and the cow reeled, sagged backward with head outstretched and eyes bulging very prominently. A picture was made of the animal at this point (see fig. 1). She then stiffened and fell to the ground on her left side with legs extended, rolled halfway over, righted herself, and got back to her feet. She then appeared fairly normal except for occasional sagging in the hind quarters. A second blood sample was taken eleven minutes after the completion of the administration of the potassium chloride. A third sample of blood was secured twenty-eight minutes later. No calcium gluconate or other therapy was employed. She was then placed in an enclosure and observed constantly for an additional period of eighty minutes. Frequent yawning was observed; otherwise the animal appeared to be normal. It was thought that the eighty minute period of constant observation was sufficient, hence it was decided to observe her at fifteen minute intervals. On return at the end of the first such interval the animal was found dead. There was little evidence of struggle on the ground where she was found. The results of the analyses of the blood samples are shown in table 3.

TABLE 2—Showing the Effect of Feeding Potassium Chloride and Potassium Citrate on the Potassium and Calcium Levels of Cow's Blood

Total amt. KCl prior to sampling (Gm.)	Total amt. of $KsC_6H_5O_7 \cdot H_2O$ prior to sampling (Gm.)	No. days receiving the salts	Plasma K mg./100 cc.	Serum Ca mg./100 cc.	Ratio K:Ca
0	0	0	16.0	11.9	1.35
0	0	0	14.5	10.7	1.35
0	0	0	16.5	10.5	1.57
1975	1675	8	24.0	10.5	2.39
3625	3025	13	16.3	10.1	1.61
4225	3625	15	18.3	10.3	1.77

A postmortem examination showed about 3 pints of fluid in the abomasum which had a

TABLE 3—Showing the Results of the Analyses of Blood Samples following the Administration of Potassium Chloride by Stomach Tube

Exp. no.	Normal blood sample			Amt.	1st blood sample after KCl			2nd blood sample after KCl		
	Plasma K mg./100 cc.	Serum Ca mg./100 cc.	Ratio K:Ca	KCl in grams	Plasma K mg./100 cc.	Serum Ca mg./100 cc.	Ratio K:Ca	Plasma K mg./100 cc.	Serum Ca mg./100 cc.	Ratio K:Ca
1	16.0	10.7	1.49	648	34.9	10.3	3.38	30.0	18.7*	1.60
2	21.5	10.5	2.04	400	29.1	10.8	2.69			
3	17.6	10.6	1.66	648	33.5	10.0	3.35	30.6	10.3**	2.97

*Calcium gluconate had been administered.
**No calcium gluconate used. Animal died.

high concentration of KCl. The presence of this amount of fluid with such a high concentration of potassium indicates that the attempt to place the administered KCl solution in the abomasum was at least partially successful. The heart was flabby and in diastole. The blood was very dark and did not coagulate. A few petichiae were on the heart over the ventricles and atria. The kidneys and bladder appeared normal, and the bladder was empty. Most of these findings are similar to those of potassium poisoning.

4) *Experiments Involving the Administration of KCl and $K_2C_2O_4 \cdot H_2O$ by Stomach Tube.*—The animal used in these experiments was a Jersey cow weighing 640 lb. She was given KCl and potassium oxalate ($K_2C_2O_4 \cdot H_2O$) twice daily by stomach tube. The salts were placed in the rumen in order to effect slow absorption more nearly like the normal condition. The amounts of the two salts administered and the results of the analyses are shown in table 4.

5) *Experiments Involving the Administration of KCl by Stomach Tube and $K_2C_2O_4 \cdot H_2O$ Intravenously.*—Calculations showed that 5.5 Gm. of $K_2C_2O_4 \cdot H_2O$ should be sufficient to lower the serum calcium of the animal used in these experiments from 10 to 6 mm./100 cc. These calculations were made, assuming a blood volume of 30 liters. The calculations were confirmed almost exactly by *in vitro* experiments.

In one experiment a normal sample of jugular blood was obtained and 5.5 Gm. of $K_2C_2O_4 \cdot H_2O$ in 150 cc. of water was injected slowly into the jugular vein. Six minutes later a second sample of jugular blood was obtained. Then 300 Gm. of KCl was administered by stomach tube, making an attempt to place this in the abomasum. The cow appeared normal in all respects throughout the experiment. Thirty-nine minutes later a third blood sample was obtained for analysis. The results of the analyses are shown in table 5.

In another such experiment, a normal jugular sample was taken and 350 Gm. of KCl was administered by stomach tube and 11 Gm. of $K_2C_2O_4 \cdot H_2O$ given intravenously. In about three minutes, the cow commenced to demonstrate the symptoms of an animal with a mild case of parturient paresis. She fell to the floor and a Shikles intravenous needle was inserted into the jugular for the obtaining of a second blood sample. She gave no evidence of objection to the insertion of the needle. Following the withdrawal of the blood sample, 100 cc. of a solution containing 5 Gm. of $CaCl_2$ was injected. She objected to withdrawal of the needle. Within three minutes the animal got to her feet. In about five minutes, her muscle commenced to quiver, and she appeared normal after this. The results

of the analyses of the blood samples are shown in table 5.

6) *Experiments Showing the Effect of Exercise on the Blood Potassium and Calcium Levels.*—In view of the known fact that exercise results in a transitory rise in blood potassium in certain animals,[4] we were interested in ascertaining if this is true of cattle. Accordingly, we carried out a series of experiments in which normal samples of jugular blood were obtained after which the animals were exercised by running, and a second sample of jugular blood was obtained. The results of the analyses of the blood samples obtained in these experiments are shown in table 6.

DISCUSSION OF RESULTS

Symptoms of tetany have developed in these experiments due to an elevation of blood potassium with no change in the calcium level from the normal, when the ratio of K:Ca exceeded 3.35 (see table 3). It has been shown in the paper[5] immediately prior to this one that animals grazing wheat are apt to show symptoms of tetany when this ratio is above 2.25. However, in all of these cases, the total serum calcium was low. By the simultaneous administration of potassium salts and oxalates, tetany was made to appear in these experiments when the K:Ca ratio was 2.62. Even in this case, the serum calcium of 9.5 mg./100 cc. was not nearly so low as in the case of animals grazing wheat (average calcium = 5.3 mg./100 cc.).

The behavior of animals in which tetany has been induced by lowering the blood calcium and raising the blood potassium is much like that of most animals grazing wheat, in that both resemble those of parturient paresis. When this tetany is induced by elevation of potassium alone, the symptoms are more spectacular, being of a convulsive nature as before mentioned. These symptoms are somewhat transitory and do not persist as they do in the case of those of cattle grazing wheat.

The higher ratio necessary to establish tetany when the serum calcium remains normal is undoubtedly due to the fact that the ratio of potassium to ionized calcium is of more importance in the maintenance of nervous equilibrium than is the ratio of potassium to total calcium.

The highest K:Ca ratio which was obtained by the feeding of potassium salts was 2.39. This was accompanied by a normal blood calcium, and hence the ratio was insufficiently high to cause any symptoms of tetany. The administration of considerable amounts of oxalate along with potassium had very little effect on blood calcium level, as will be obvious from an examination of table 4. Cattle must have a remarkably labile calcium system. It takes many times the calculated amount of oxalate to produce the expected lowering of blood calcium. In the experiments reported in table 5, the amount of oxalate used should have pro-

TABLE 4—Showing the Effect of the Daily Administration of Potassium Chloride and Potassium Oxalate by Stomach Tube

Date 1946	Amt. KCl (Gm.) a. m.	Amt. KCl (Gm.) p. m.	Amt. $K_2C_2O_4$ (Gm.) a. m.	Amt. $K_2C_2O_4$ (Gm.) p.m.	Plasma K mg./100 cc.	Serum Ca mg./100 cc.	Ratio K:Ca
10/23	150	150	15	15	15.4	10.6	1.45
10/24	150	None	15	None			
10/25	150	150	15	15	16.1	9.3	1.73
10/26	150	150	15	15			
10/27	150	150	15	15			
10/28	150	150	15	15			
10/29	150	75	15	15			
10/30	150	150	15	15	18.8	10.1	1.86
10/31	150	150	15	15			
11/1	225	225	30	30	21.8	10.2	2.14
11/2	225	225	30	30			
11/3	225	225	30	30			
11/4	225	225	30	30			
11/5	225	225	30	30			
11/6	225	225	30	30	22.8	10.7	2.13
11/7	225	225	30	30			
11/8	225	225	30	30			
11/9	225	225	30	30			
11/10	225	None	15	None			
11/11	225	225	30	30	18.8	10.5	1.79
11/12	225	225	30	30			
11/13	225	None	15	None			
11/14	225	None	15	None			
11/15	225	None	15	None			
11/16	225	None	15	None		9.99	

TABLE 5—Showing the Effect of the Intravenous Injection of Potassium Oxalate and the Oral Administration of Potassium Chloride on Plasma Potassium and Serum Calcium Levels

Wt. KCl given (Gm.)	Wt. $K_2C_2O_4$ H_2O given (Gm.)	Normal blood sample			1st sample following administration of salts			2nd sample following administration of salts		
		Plasma K mg./100 cc.	Serum Ca mg./100 cc.	K:Ca Ratio	Plasma K mg./100 cc.	Serum Ca mg./100 cc.	K:Ca Ratio	Plasma K mg./100 cc.	Serum Ca mg./100 cc.	K:Ca Ratio
300	5.5	17.1	10.3	1.66	18.1	10.1	1.80	19.0	10.1	1.88
350	11.0	13.7	11.8	1.17	24.9	9.5	2.62	21.6	16.1*	1.34

*5 Gm. KCl given prior to taking sample.

duced about five times as great a lowering of blood calcium as was observed. We have observed this relationship in a number of other experiments not herein reported. The transitory nature of blood potassium increases is also apparent from an examination of this table. On Nov. 10, 1946, the potassium salts were omitted in the evening feeding, and the plasma potassium was not as elevated the following morning as it had been in previous experiments. We have noticed this same condition in connection with the experiments involving the feeding of potassium salts.

TABLE 6—Showing the Effect of Exercise on the Plasma Potassium Content and the Serum Calcium Content of Cows

Exp.	Before Exercise			After Exercise		
	Plasma K mg./100 cc.	Serum Ca mg./100 cc.	K:Ca Ratio	Plasma K mg./100 cc.	Serum Ca mg./100 cc.	K:Ca Ratio
1	17.4	10.7	1.62	16.9	10.6	1.59
2	22.9	11.4	2.01	21.3	11.3	1.88
3	24.0	10.5	2.28	22.3	10.8	2.06
4	19.8	10.3	1.92	17.9	10.3	1.74
5	19.0	10.9	1.74	17.6	10.5	1.68

The experiments on the effect of exercise on blood potassium were conducted because it is well known that the excitement, exercise, etc., involved in the shipping and handling of cattle which have been grazing wheat, apparently, often precipitates the attack of tetany. Exercise has been shown to cause a temporary rise in blood potassium in some animals.[4,5] Our experiments show that exercising cattle for a short period of time actually results in a slight decrease in blood plasma potassium.

SUMMARY

1) It is possible to raise the K:Ca ratio of the blood of cattle considerably by the feeding of potassium salts, but in the absence of any lowering of calcium no tetany symptoms have been observed.

2) Raising of plasma potassium by the oral administration of potassium salts, and lowering of calcium by intravenous injec-

tion of $K_2C_2O_4 \cdot H_2O$, produce tetany when the K:Ca ratio is sufficiently altered. Oxalates do not have the calculated effect on blood calcium *in vivo*.

3) It is possible to raise the blood potassium of cattle to a considerable extent by administering large amounts of KCl by stomach tube in such a way as to deposit the KCl in the abomasum. When the plasma potassium to serum calcium ratio is sufficiently high (about 3.35), marked tetany symptoms are displayed.

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The Value of Supplementary Vitamins for Calves Raised Under Farm Conditions

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THE REPORTED[3,4,7,8] beneficial effects of vitamin supplementation in preventing scours and reducing the death rate among young calves have not been confirmed by similar studies[2,5,6] made with calves raised in college and experiment station herds. The value of supplementary vitamins for calves raised under less favorable conditions, conceivably, might be different.

The data reported in this paper contain the results of a large-scale field trial to determine the value of supplementary vitamins for calves raised under farm conditions.

PROCEDURE

Carefully selected state institution and farmer herds in various sections of New York State were chosen as coöperators in this field study. Reliability and accuracy in following directions and keeping records, number of calves raised, and general feeding and management conditions were used as criteria in selecting the coöperating herds. Each herd was rated good, fair, or poor on calf feeding and management practices, and on the general herd feeding and management practices. Detailed information on the system of raising calves, housing facilities, sanitation, and other pertinent factors were considered in arriving at these ratings. In a majority of cases, the calves were raised according to the Cornell limited, whole milk, dry starter system.

Each coöperator was supplied with two kinds of tablets which were colored differently. One kind contained the vitamins, and the other contained inert material. The composition of the tablets was not known by the coöperators while the experiment was in progress. Daily observations of each calf as to the occurrence and severity of scours or others diseases were recorded by each coöperator on a standard record form. Each calf was graded on physical condition and appearance at 30 days of age. Visits were made to each herd at frequent intervals, by the project leaders, to assure close supervision of the study. Any coöperator failing to comply with the conditions of the experiment was excluded.

Experiment 1.—In April, 1945, an extensive field trial was started with 48 coöperators. Every calf received 1 tablet daily from birth to 30 days of age. Alternate calves received pills containing vitamins, and the other calves received the inert kind. The vitamin tablets contained the following daily dose: 5,000 I.U. of vitamin A, 1,000 U.S.P. units of vitamin D, 250 mg. of vitamin C, and 100 mg. of niacin. This trial was conducted for a period of eighteen months to include different climatic conditions.

Results.—The results are presented and discussed, according to the feeding and management programs followed.

Good Calf Feeding and Management Conditions.—The results of feeding vitamins to calves raised under good feeding and management conditions are summarized in table 1. These data were collected in nine Holstein-Friesian, two Guernsey, and two Ayrshire herds. The limited number of Guernsey and Ayrshire herds preclude a comparison of results between breeds. There were no distinct seasonal or treatment differences in the incidence of scours, the average age at which scouring began, the duration of scouring, or the apparent physical condition of the calves at 30 days of age (table 1).

Fair Calf Feeding and Management Conditions.—The incidence of scours among calves raised under fair feeding and management conditions was somewhat higher during the winter months than during the other seasons, as shown in table 1. Although the difference is not conclusive, 34.1 per cent of the fall control calves and 55.3 per cent of the winter control calves scoured, as compared to 21.4 per cent and 46.9 per cent cases of scours in the vitamin supplemented calves.

Only 16 per cent of the Guernsey calves re-

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ceiving the vitamin pills exhibited scours, as compared to 35 per cent of the control Guernsey calves. These data are suggestive of a beneficial effect of vitamin supplementation. It should be pointed out, however, that the number of calves involved is small and represents results in only three herds. Little difference was noted between the control calves and the vitamin supplemented calves of the Holstein-Friesian breed. Three calves of the control group and 5 calves of the vitamin supplemented group died during the course of the experiment. The data shown in table 1 were obtained in one Ayrshire, one Jersey, 18 Holstein-Friesian and three Guernsey herds.

Poor Calf Feeding and Management Conditions.—Data collected in ten Holstein-Friesian herds, one Guernsey, and one Ayrshire herd are summarized in table 2. It is apparent from these data, based on 147 control calves and 162 vitamin supplemented calves raised under poor feeding and management conditions, that the additional vitamins did not visibly benefit the young calf.

Experiment 2.—Inasmuch as the newborn calf is usually deficient in vitamin A, and the vitamin A content of colostrum is extremely variable, it has been suggested that a massive dose of vitamins fed at birth might afford the young calf some measure of protection against scours. Recent studies of Eaton *et al.*[1] have shown that the plasma vitamin A was maintained at a level higher than normal for a period of forty-two days by the feeding of 250,000 I.U. of vitamin A at birth. A field trial, similar to the one described in experiment 1, was undertaken during the winter of 1946. Sixteen coöperators were selected for this study. The procedure was identical to that followed in experiment 1, except that the vitamin supplemented calves received a single dose of 250,000 I.U. of vitamin A, 500 mg of niacin, and 50,000 U.S.P. units of vitamin D at birth and none thereafter.

Results.—The results are presented and discussed, according to the calf feeding and management conditions on the coöperating farms.

Good Calf Feeding and Management Conditions.—Data were obtained in two Guernsey, two Ayrshire, and four Holstein-Friesian herds. No differences were noted among the groups regarding the incidence, beginning age,

TABLE 1—The Effect of Vitamins on Scours and General Appearance of Calves Raised under Good, Fair, or Poor Feeding and Management Conditions

	Control Calves						Vitamin-Supplemented Calves					
	No. of calves	Incidence of		Condition*			No. of calves	Incidence of		Condition*		
		Scours (%)	Death (No.)	Good (%)	Fair (%)	Poor (%)		Scours (%)	Death (No.)	Good (%)	Fair (%)	Poor (%)
GOOD FEEDING AND MANAGEMENT CONDITIONS												
All Breeds												
Spring	23	17.4	..	91.3	8.7	...	25	20.0	1	95.8	4.2	...
Summer	28	21.4	..	96.4	3.6	...	26	19.2	1	100.0	...	...
Fall	37	18.9	..	91.9	8.1	...	43	14.0	..	93.0	2.3	4.7
Winter	53	20.8	..	88.7	9.4	1.9	54	20.4	..	87.0	11.1	1.9
All Seasons												
Ayrshire	14	21.4	..	100.0	...	...	12	33.3	..	100.9	...	...
Guernsey	67	13.4	..	95.5	4.5	...	70	10.0	..	97.1	2.9	...
Holstein-Friesian	60	26.6	..	85.0	13.3	1.7	66	24.2	2	85.9	9.4	4.7
All Breeds												
All seasons	141	19.9	..	91.5	7.8	0.7	148	18.2	2	93.8	4.2	2.0
FAIR FEEDING AND MANAGEMENT CONDITIONS												
All Breeds												
Spring	70	24.3	1	84.1	15.9	...	75	22.7	..	84.0	14.7	1.3
Summer	32	21.9	1	81.3	18.7	...	26	23.1	..	88.5	11.5	...
Fall	41	34.1	..	61.0	36.6	2.4	42	21.4	2	72.5	27.5	...
Winter	85	55.3	1	58.4	36.9	4.7	96	46.9	4	65.2	32.6	2.2
All Seasons												
Ayrshire	7	42.8	..	28.5	71.5	...	6	50.0	..	16.7	66.6	16.7
Guernsey	20	35.0	1	84.2	15.8	...	25	16.0	..	92.0	8.0	...
Holstein-Friesian	197	36.5	2	70.0	27.4	2.6	201	33.3	5	74.6	23.9	1.5
Jersey	4	75.0	..	...	75.0	25.0	7	42.9	1	66.6	33.4	...
All Breeds												
All seasons	228	37.3	3	70.2	27.5	2.3	239	32.2	6	75.1	23.3	1.6
POOR FEEDING AND MANAGEMENT CONDITIONS												
All Breeds												
Spring	43	44.1	5	84.2	15.7	...	48	37.5	1	87.2	13.8	...
Summer	25	16.0	..	92.0	8.0	...	26	15.4	1	100.0	...	...
Fall	33	42.4	..	78.1	15.9	6.0	32	37.5	1	90.0	10.0	...
Winter	46	67.3	7	61.5	25.6	12.8	56	67.8	8	71.7	23.9	4.3
All Seasons												
Ayrshire	7	42.9	..	85.7	14.3	...	5	20.0	..	80.0	20.0	...
Guernsey	3	33.3	1	100.0	...	...	5	40.0	1	75.0	25.0	...
Holstein-Friesian	137	46.7	11	77.7	17.5	4.8	152	44.7	10	83.8	14.8	1.4
All Breeds												
All seasons	147	46.2	12	78.6	17.0	4.4	162	44.4	11	83.4	15.2	1.4

*Condition of calf at 30 days of age.

or duration of scours as shown in table 2. The percentages of the calves grading good, fair, or poor at 30 days of age were approximately the same in both treatments.

Fair Calf Feeding and Management Conditions.—The effects of a single massive dose of vitamins A, D, and niacin on the health of young calves raised under fair feeding and management conditions are summarized in table 2. Fewer cases of scours were noted among the vitamin supplemented calves, although the difference was too small to warrant a conclusion.

Poor Calf Feeding and Management Conditions.—The ineffectiveness of the vitamin supplements, in preventing scours or reducing the duration of scours in Holstein-Friesian calves raised under poor feeding and management conditions, is evident from the results summarized in table 2.

DISCUSSION

From the results of the field studies reported, it is evident that feeding extra vitamins to newborn calves raised under farm conditions of good, fair, or poor feeding and management did not consistently reduce the incidence or duration of scours. There was no noticeable improvement in the general appearance and condition of the calves up to 30 days of age. These results are in agreement with studies made by Norton *et al.*[6] in the Cornell University herd, by Hibbs and Krauss[2] in the Ohio Experiment Station herd, and by Nevens and Kendall[5] in the Illinois Experiment Station herd. The writers are inclined to the view that the routine feeding of vitamin tablets to calves raised under New York conditions is not a panacea for calf ills and should not be recommended as a substitute for good feeding and management practices. Moreover, this view seems to be justified because of the greater incidence of scours and higher death losses among the calves subjected to poor feeding and management practice.

It should not be inferred that the results reported herein preclude the possibility of specific vitamin or other nutritional deficiencies occurring in individual calves. In such cases, it may be expedient to resort to vitamin therapy. Nevertheless, while it is known that a severe deficiency of vitamin A,

TABLE 2—The Effect of Vitamins on Scours and General Appearance of Calves Raised under Good, Fair, or Poor Feeding and Management Conditions.

	Control Calves						Vitamin-Supplemented Calves					
	No. of calves	Incidence of		Condition*			No. of calves	Incidence of		Condition*		
		Scours (%)	Death (No.)	Good (%)	Fair (%)	Poor (%)		Scours (%)	Death (No.)	Good (%)	Fair (%)	Poor (%)
GOOD FEEDING AND MANAGEMENT CONDITIONS												
All Breeds												
Spring	17	11.8	..	82.4	17.6	...	23	21.7	..	100.0	...	...
Summer	7	...	..	100.0	...	...	10	...	..	100.0	...	...
Fall	11	18.2	..	100.0	...	...	11	9.1	..	100.0	...	...
Winter	21	19.0	1	90.0	10.0	...	27	18.5	..	81.5	7.4	11.1
All Seasons												
Ayrshire	10	20.0	..	100.0	...	...	11	9.1	..	100.0	...	...
Guernsey	21	19.0	1	90.0	10.0	...	29	17.2	..	89.7	3.4	6.9
Holstein-Friesian	25	8.0	..	96.0	4.0	...	31	16.1	..	93.5	3.2	3.3
All Breeds												
All seasons	56	14.3	1	90.9	9.1	...	71	15.5	..	93.0	2.8	4.2
FAIR FEEDING AND MANAGEMENT CONDITIONS												
All Breeds												
Spring	4	...	..	100.0	...	...	5	...	..	100.0	...	...
Summer	3	...	..	100.0	...	...	5	20.0	..	100.0	...	...
Fall	7	42.9	..	85.7	14.3	...	6	16.7	..	100.0	...	...
Winter	10	50.0	..	70.0	30.0	...	13	23.1	1	75.0	25.0	...
All Seasons												
Holstein-Friesian	14	35.7	..	92.9	7.1	...	17	17.6	1	87.5	12.5	...
Jersey	10	30.0	..	70.0	30.0	...	12	16.7	..	91.7	8.3	...
All Breeds												
All seasons	24	33.3	..	83.3	16.7	...	29	17.2	1	89.3	10.7	...
POOR FEEDING AND MANAGEMENT CONDITIONS												
Holstein-Friesian												
Spring	18	50.0	3	66.6	34.4	...	24	41.7	1	91.3	...	8.7
Summer	9	33.3	..	88.9	11.1	...	12	41.7	..	41.7	50.0	8.3
Fall	2	50.0	..	50.0	...	50.0	3	33.3	..	33.3	66.7	...
Winter	17	47.1	..	64.7	35.3	...	17	41.2	1	81.3	18.7	...
All Seasons												
Holstein-Friesian	46	45.7	3	69.7	27.9	2.4	56	41.1	2	68.5	20.4	5.6

*Condition of calf at 30 days of age.

and possibly other vitamins, will cause diarrhea, care must be exercised in assuming that scours in young calves is a definite indication of vitamin deficiency. It is difficult to determine whether a particular case of scours is due to nutritional deficiency, bacterial infection, or faulty feeding and management practices.

In the herds studied, somewhat less trouble with calf scours was experienced during the summer months. This is to be expected in view of the changeable climatic conditions which prevail in New York during the late fall, winter, and spring months.

In studies of this nature, the opinions of the coöperating dairymen, although not entirely objective, should be considered. Preference for the tablets containing the vitamins or those containing inert material was divided about equally.

SUMMARY AND CONCLUSIONS

The results are reported of a large-scale field trial to determine the value of supplementary vitamins for calves raised under farm conditions. Forty-eight herds, largely Holstein-Friesian, located throughout New York State, were selected as coöperators. These herds were classified into three groups: good, fair, or poor, according to the calf feeding and management conditions on each farm.

The criteria used in evaluating the results were the frequency, duration, and age at which scours occurred, and the physical appearance and condition of the calves at 30 days of age. Data were accumulated on 1,347 calves.

In experiment 1, alternate calves received daily, from birth to 30 days of age, a tablet containing 5,000 I.U. of vitamin A, 250 mg. of vitamin C, 1,000 U.S.P. units of vitamin D, and 100 mg. of niacin. The other calves received a placebo capsule. There were 181 cases of scours observed in the 516 calves of the control group and 176 cases of scours in the 549 vitamin supplemented calves.

Alternate calves in experiment 2 received a tablet at birth containing 250,000 I.U. of vitamin A, 50,000 U.S.P. units of vitamin D, and 500 mg. of niacin. The other calves received a placebo capsule. Thirty-seven cases of scours in the 126 control calves and 39 cases of scours in the 156 vitamin supplemented calves were noted.

It is concluded that feeding extra vitamins to newborn calves, raised under farm conditions of good, fair, or poor feeding and management, did not materially reduce the incidence or duration of scours, nor was there any apparent improvement in the general appearance and condition of the calves up to 30 days of age.

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Studies on Bovine Gastrointestinal Parasites. X.

The Effects of Nodular Worm (*Oesophagostomum Radiatum*) on Calves During the Prepatent Period

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IN A FORMER paper,[1] the results of the effects of the stomach worm (*Haemonchus contortus*) on the weights of calves during the larval or prepatent period of the life cycle were reported. It was found that in most instances there was little or no gain during this period. In an earlier paper,[2] it was shown that a definite anemia was developed during the prepatent period. However, it was found that the effects of the large stomach worm were much less severe than had been indicated by field observations. Observations were also recorded on 6 animals that died at intervals varying from thirteen to thirty-three days after infection with nodular worm and stomach worm larvae. The results of some experiments with pure infections of the nodular worm on calves during the prepatent period are reported herein.

LITERATURE

Several references were mentioned in a former paper.[1] Additional papers are of interest in this connection, because they report severe symptoms of parasitism during the prepatent period of *Chabertia ovina* infections in sheep[3, 4] and severe diarrhea and emaciation during the prepatent period of hookworm (*Bunostomum phlebotomum*) infections in calves.[5]

METHODS OF PROCEDURE

All calves used in our experiments were secured within two days after birth from the Louisiana State University dairy department or from local dairymen and placed in individual cages in the animal building of the veterinary science department. The cages had wire floors and were cleaned and washed twice a week to prevent the introduction or spread of any existing infection in the building. The calves were fed milk for the first three to four months, hay that had been cut from ungrazed ground, a commercial calf concentrate, and city water. Fecal examinations were made at regular intervals in order to determine the absence of infection and to trace the course of experimental inoculations.

RESULTS

Calves 149 and 150 were purebred Holstein-Friesian males obtained from the L. S. U. dairy department and were born on Oct. 13 and 17, 1944, respectively. They were raised parasite free, as was determined by several fecal examinations. Both animals received equal amounts* of the same suspensions of infective larvae cultured from a calf with a pure infection of nodular worms on the following dates: Feb. 16, 21, March 1, 5, 14, 19, and 21, 1945 (*See* inoc., fig. 1). The inoculation dates are shown by short upright lines, the minimum prepatent period by the first open box, and the maximum prepatent period following the last inoculation by the last box at the end of the line. On March 10, calf 149 developed a severe diarrhea which lasted in its worst form for nineteen days (fig. 1). Calf 150 had a similar diarrhea for fourteen days (fig. 1). Both animals continued to have an intermittent diarrhea for about eighteen days as indicated. The diarrhea associated with the acute symptoms of parasitism, which develops during the

*In connection with administering a definite number of larvae, I have never been able to work out a satisfactory method of making a count. It is obviously impossible to accurately count every individual larva in the 25 oz. or so of the suspension used in some experiments. If it were possible to keep the larvae still long enough under the microscope, I doubt if one would live long enough to complete the job. It is not possible to get an even suspensin from which to take a few drops. If the number is counted in such a few drops and it be slightly incorrect, it must be multiplied by the total number of drops in the amount administered. I believe these results would be so inaccurate that it would be of little value. I have discussed this question with others who make experimental inoculations with parasites and they are of the same opinion.

The object of these experiments was to show as accurately and as completely as possible the effects of the larvae on the calves in comparison with the effects of the adult parasites. I do not see how I could have killed the calves to recover the adults in order to state how many were present and then have had the calves with which to determine the effects of the adults. I have submitted, I think, numerous and high egg counts as evidence of the success of the inoculation, described the effects on the animals and furnished weights in support of the fundamental conclusions offered in the paper.

larval period, is not only watery but at times contains blood and small blood clots, and small shreds and strings of mucus. There is always present a disagreeable odor, varying in degree with the severity of symptoms. This is in contrast to the type of diarrhea mentioned in a former paper,[6] which is associated with the development of immunity. Such diarrhea is merely a softening of the manure, and offensive odor or blood is absent.

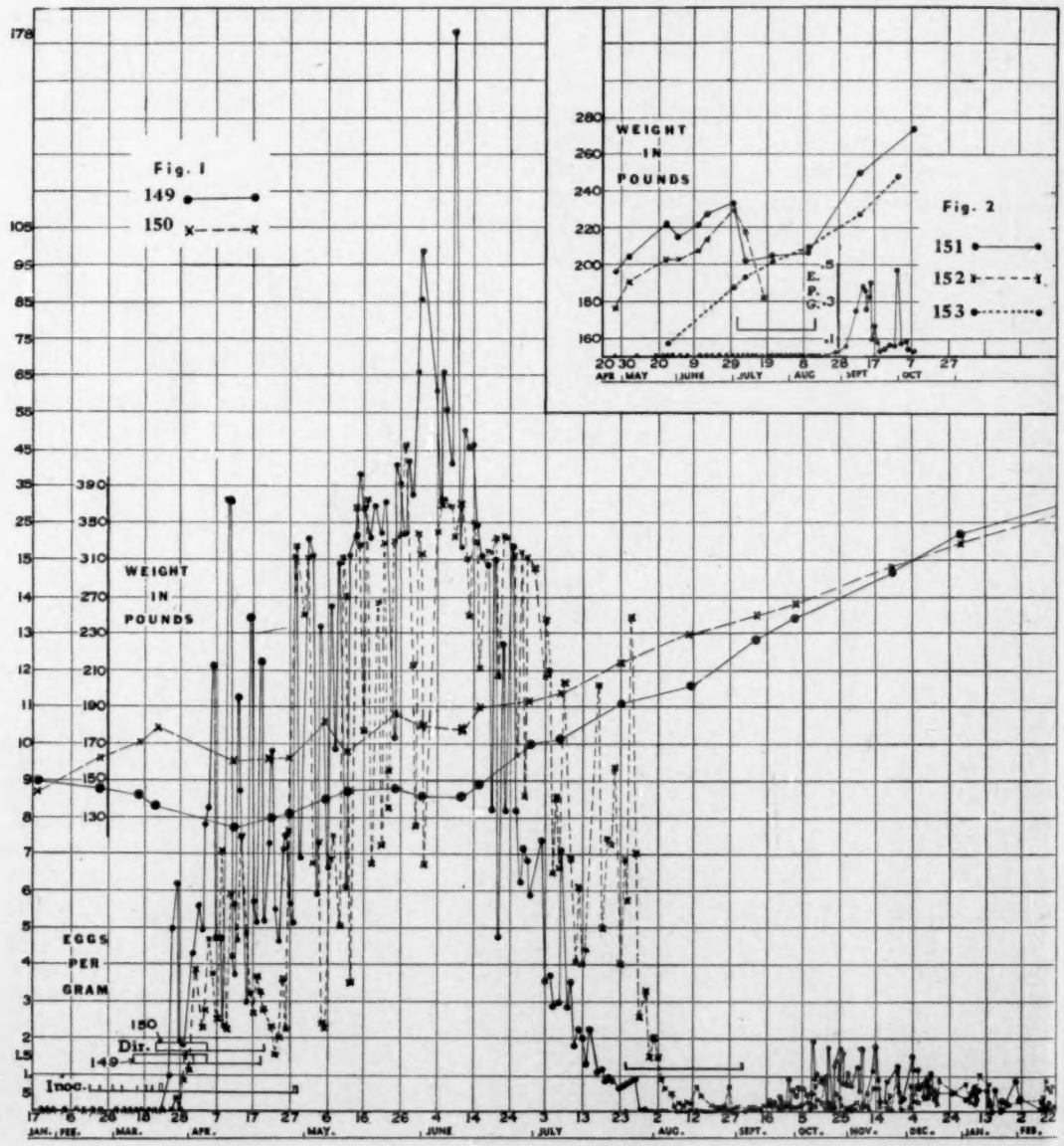
The first nodular worm eggs appeared in the feces of calf 149 on March 23, and in the feces of calf 150 on March 24 (fig. 1). It will be noted that the egg counts remained low until about April 7. This was followed by a gradual increase, until maximum counts were obtained in the first part of June. The egg counts of calf 149 decreased sharply about the following dates: June 15, 25, and July 12. By July 27, the egg counts were almost zero. After inoculation with additional larvae on July 24 (fig. 1), there was only a slight increase, beginning about two weeks after the maximum prepatent period of about forty days. These results are in accord with our previous observations that, in immune calves, there is often a slight increase in the egg counts, and the increase often occurs after the maximum prepatent period determined for nonimmune calves. An examination of the egg counts of calf 150 shows that they remained at a high level until July 25, when there was a rapid decrease within a week to less than 1 egg per gram of sediment. They remained at this very low level until the end of the experiment, March 11, 1946, even though the calf was reinoculated on July 24, 1945. This date, followed by the usual prepatent period, is indicated in figure 1.

The weights of both animals were taken during the experiment and are shown graphically in fig. 1. It will be noted that calf 150 gained steadily until March 21, thirty-two days after receiving the first inoculation, at which time it developed a severe diarrhea and began to lose weight. Between March 21 and April 27, its weight decreased from 178 to 162 lb. During this time, it became very weak and emaciated and, at times, lacked the strength to get up. Because of this weakness, it was considered inadvisable to take the calf to the scales to be weighed. Part of the time it refused to eat and drink, or ate and drank only a fraction of the usual amount. Following the early part of April, after the egg count began to increase, its strength and general condition rapidly improved, and its weight began to increase slightly but irregularly. These observations indicate that after the larvae had completed their development within the wall of the intestine, the calves were able to tolerate the presence of the adult worms in the lumen of the intestine without severe symptoms. It will be noted that after the development of immunity, as evidenced by the decreased egg count (fig. 1), there was a more rapid gain in weight than during the period of high egg count. We do not know how much of this increased gain was due to the recovery of normal function resulting from healing of the damage done by the larvae while they were in the wall of the intestine, and how much was due to the elimination of the adults. If we had been conducting treatment experiments to remove the adults and had based the results on the egg counts we might have concluded that all the gain in weight was the result of treatment, but after observing the animals and studying sections of the intestinal wall from calves killed while the parasitism was in the larval period, we do not believe that such a conclusion is justified.

Calf 149 did not make regular gains during February and early March (fig. 1). It was fed cottonseed hulls in an attempt to find out if a low plane of nutrition would influence the level of parasitic infection. An examination of the resulting weights (fig. 1) shows a great similarity to those of calf 150 in that during the larval period there was a loss, and then as the larvae matured (as evidenced by the increasing egg count), the weight increased. The general physical condition of emaciation and weakness was very similar to that of calf 150. Also, while the egg count was increasing and during the period it was highest, its general physical condition showed a

marked improvement, as in the case of calf 150. The egg count increased steadily until about the middle of June when a marked decrease began. There was also a decrease in the number of eggs about June 24 and July 10. The egg count then remained at a low level until the end of the experiment, remaining relatively low following reinoculation on July 24, 1945. A slight rise in the egg count did occur after the inoculation on July 24, after the usual prepatent period for the nodular worm (fig. 1). It is not uncommon for either or both of these results to occur in immune calves when reinoculated.

Both calves 149 and 150 were removed on March 11 from the animal building where they had been kept under very favorable conditions, in so far as feed and weather were concerned, to the pasture used for parasite experiments. On April 15, calf 149 died due possibly to its inability, resulting from incomplete recovery, to live under the outside unfavorable weather and grazing conditions. Calf 150 lived but lost considerable in general condition.



Calves 151 and 152 were purebred Jersey males born Nov. 11, 1944. They were given equal amounts of the same suspension of nodular worm larvae by mouth on July 2, 1945. Symptoms began to develop very quickly in both these animals (fig. 2). Calf 151 lost 32 lb. and calf 152 lost 12 lb. within five days after inoculation; both animals developed a very severe diarrhea beginning seven days after inoculation. On July 16, fourteen days after inoculation, calf 152 was unable to hold up its head and had lost 48 lb. On June 30, it weighed 230 lb. and on July 16, weighed 182 lb. Since it was in such a weakened condition, and because intestinal tissues were desired to study the larvae in the intestinal wall, calf 152 was slaughtered for this purpose. At postmortem examination, the anterior half of the small intestine was normal in appearance but the posterior half showed numerous inflamed areas, varying from pinpoint size to ⅛ in. in diameter. Three areas 1½ to 2 ft. long showed severe catarrhal enteritis in the posterior portion of the small intestine. In the large intestine, an area about 3 ft. long in the region of the ileocecal valve showed inflammation and enteritis. Many immature nodular worms were recovered from the posterior half of the small intestine and from the large intestine. Calf 151 ceased to lose weight and by the end of the prepatent period had begun to gain (fig. 2). Its general condition improved also, and following the end of the prepatent period, it gained rapidly in weight and general condition. The first nodular worm eggs were recovered from the feces of calf 151 on August 27, fifty-seven days after inoculation. This is an unusually long prepatent period. However, we have noted in other animals that maturing of the larvae has often been delayed if the calf showed severe symptoms of parasitism, or if immunity developed. A very low egg count was obtained, another result which often follows severe symptoms. Calf 153 was the control calf during this experiment, and it made regular gains during the experiment (fig. 2).

On October 8, calf 151 was removed from the animal building to the pasture used for such experiments. Its general condition continued to improve during the following month and on November 13, a fecal examination showed only a small number of nodular worm eggs. During the following month, the condition of the pasture deteriorated because of winter conditions, and the calf began to lose weight rapidly. On Dec. 14, 1945, it was found dead. Since it had been dead several hours, no postmortem examination was carried out. There were 4 other animals in the pasture at the same time which, although they lost flesh, came through the winter in fair condition. It would seem from our observations that calf 151 had possibly received a severe injury during the larval period of the parasites from which it had not sufficiently recovered after five months to be able to withstand moderately severe weather and grazing conditions.

Sections were cut from four regions of the small intestine and from the cecum of calf 152. One hundred transverse sections (the equivalent of 1 mm. in intestinal length) and approximately 2 cm. in length from each of three regions of the posterior 19 ft. of the small intestine showed from 5 to 12 larvae upon microscopic examination. No larvae were found to have penetrated into the tissue in the anterior portion at a point 20 ft. posterior to the pylorus. Neither were any larvae found to have penetrated at a point 8 in. posterior to the ileocecal valve.

Calf 155 was a grade Jersey male born March 19, 1945, and was obtained from the L. S. U. dairy research department when a few hours old and was kept under conditions free of parasitic infection, as was demonstrated by 12 negative fecal examinations between May 16 and August 4, including five daily examinations from July 31 to August 4. On July 31, 1945, calf 155 was given a large number of infective nodular worm larvae by mouth. On August 2, the second day after inoculation, the calf drank about two thirds the usual amount of water, refused to eat the grain concentrate at night, and ate little or no hay during the day. During the three following days, it drank one fourth to one half the usual amount of water, ate little or no hay, and

refused the grain concentrate.

On August 6, six days after inoculation, this calf was slaughtered to obtain tissues for histologic study. At the postmortem examination, the small intestine was found to be inflamed throughout almost its entire length. Only the anterior 3 to 4 ft. appeared normal. There were numerous pinpoint and pinhead size hemorrhagic areas, and scrapings from eight different regions showed from 1 to 3 larvae under a 40-mm. cover glass. The large intestine, the cecum, and approximately 1 ft. posterior to the ileocecal valve showed much hemorrhage in large spots and elongated areas ⅛ in. in diameter and many pinpoint size spots. Three scrapings from different points showed from 1 to 4 larvae each.

Serial sections were cut from eight regions of the small intestine of calf 155. The blocks of tissue were cut 10μ thick and in such a manner as to extend approximately 2 cm. transversely across the intestine. An examination of the sections showed from 5 to 14 larvae per 100 sections (1 mm. in length of the intestine) in the regions examined from the posterior 15 ft. At a point 22 ft. anterior to the ileocecal valve, only 1 and 2 larvae were found to have penetrated the tissues. Ninety to 100 sections were examined at points 1, 5, and 20 ft. posterior to the pylorus without finding any larvae within the tissues, even though most of this region showed considerable gross inflammation.

SUMMARY

Data are presented on 5 calves that were inoculated with pure cultures of infective nodular worm larvae under parasite controlled conditions, which show that the symptoms generally associated with parasitism develop during the prepatent period.

Diarrhea developed as early as the seventh day and as late as the seventeenth day after inoculation and continued as long as nineteen days. There followed periods of intermittent diarrhea, lasting as long as eighteen days in some of the experiments.

The appetite began to be affected as early as two to five days after receiving the infective larvae.

Loss in weight, severe emaciation, and general weakness were associated with the diarrhea. The general condition improved with the beginning of egg production by the parasites.

Two animals developed an immunity, as was evidenced by a rapid drop in egg count and failure to become reinfected upon reinoculation.

The effects of severe parasitism seem to persist for a long time in some individual animals, as suggested by the death of 2 animals under adverse environmental conditions five and twelve months after the development of symptoms.

The improvement in general condition, following the maturing of larvae, suggests that treatment at this time had less influence than is generally supposed, while the improvement was mainly the result of the natural course of the infection. However, the removal of adult parasites by administering a suitable anthelmintic to infected animals that are able to withstand treatment is extremely desirable, since, in so doing, the egg and larval population of the premises is reduced. This reduces the danger of loss from heavy larval infection of the next generation of calves and the reinfection of the present generation of calves.

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Studies on Bovine Gastrointestinal Parasites. XI.

The Life Cycle of the Hookworm (*Bunostomum Phlebotomum*) in the Calf

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THE HOOKWORM *Bunostomum phlebotomum* is a common parasite of cattle in the United States, particularly in the southern states. It is usually found in small numbers even in healthy animals that are brought to slaughter. The parasites are to be found in the anterior portion of the small intestine, and a few in the abomasum. This report deals primarily with observations on mode of infection, prepatent period, and symptoms.

LITERATURE

An account of this species in the field was given by Conradi and Barnette[1] who recorded observations on pathogenicity, postmortem examination, studies on the eggs, larvae, control methods, and treatment. Schwartz[2] recorded data on the preparasitic larval stages. He concluded, as a result of using the method of Goodey, that they did not penetrate the skin. Mayhew[3] reported that infection takes place by way of the skin. A preliminary report on part of the data included in this paper is to be found in Mayhew[4] in which skin penetration is further demonstrated as a mode of infection. Sprent[5] recorded observations on reproduction, preparasitic larval stages, and parasitic larval stages, and demonstrated infection by way of the skin, and recorded observations on the preparasitic larval stages.[6]

Cameron[7] failed to demonstrate skin penetration in the life cycle of *Bunostomum trigonocephalum*, the species of hookworm parasitic in sheep. Beller[8] reported cutaneous infection of 1 sheep with *B. trigonocephalum*. Lucker and Neumayer[9] reported that *B trigonocephalum* was pathogenic and demonstrated cutaneous as well as oral infection. Ortlepp[10, 11] studied the life cycle of *Gaigeria pachyscelis*, another species of hookworm parasitic in sheep, and demonstrated skin penetration of the larvae.

METHODS OF PROCEDURE

Calves were obtained for these experiments from the Louisiana State University dairy department when they were under 24 hours old and placed in individual cages in the animal building of the Veterinary Science Department. The system of management and feeding employed in caring for all calves for experiments with parasites over a period of years has been found to exclude nonexperimental infections. The quarters were cleaned twice a week, the hay fed was cut from ground that had not been grazed, the grain concentrate was a well-known commercial brand, and the water was from the Baton Rouge city water supply.

The skin inoculations were made by placing the infective larvae on the unclipped skin in an area 6 to 8 in. in diameter and covering the area with the hands for twenty to thirty minutes in order to prevent too rapid drying of the area and to provide some heat in an attempt to simulate the conditions created as the animal lies down. Large numbers of larvae were concentrated by centrifuging in 10 to 15 cc. of water before applying to the skin.

EXPERIMENTAL

Skin Inoculations.—Calf 159 was a purebred Holstein-Friesian male, born Oct. 11, 1945. On May 26, 1946, a large number of infective larvae cultured from calf 158 was placed on the skin of the left flank. The calf was over 7 months old at the time of inoculation and had been proved free from infection by 11 fecal examinations. The animal was then restrained in a stanchion so constructed that he could not reach any part of the body with his nose, and kept securely fastened until eggs appeared in the feces. The first eggs appeared on July 22, 1946, fifty-seven days after the application of the larvae (fig. 1a). A total of 41 fecal examinations previous to the appearance of eggs were consistently negative. The egg count, it will be noted (fig. 1a), remained low for about a week, then increased to a maximum of 20 eggs per gram of sediment. This calf did not show any diarrhea or other symptoms of parasitism during the entire experiment. He was kept under controlled conditions of parasitic infection during the next thirteen months, and a total of 83 positive fecal examinations showed only typical hookworm eggs.

Calf 160 was a purebred Holstein-Friesian male, born Oct. 5, 1945. A large number of infective hookworm larvae was placed on the skin of the right flank on April 23, 1946, and the calf placed in a stanchion. On June 14, he escaped from the stanchion, seven weeks and four days after the application of the

larvae to the skin. He was 6 months and 18 days old when the larvae were applied to the skin, and 66 fecal examination, made before the appearance of eggs in the feces, were consistently negative. The first eggs appeared on June 25, 1946 (fig. 1a), sixty-six days after the application of the larvae to the skin. On May 24, thirty-one days after inoculation, the calf developed a severe diarrhea which continued till June 3. The feces contained more or less blood which was evident on the surface of the sample and appeared in the tubes during the process of centrifuging. There was also a very disagreeable odor which was evident upon entering the room where the calf was kept.

Further applications of larvae were made as follows: October 19, 4:30 p. m.; October 21, 8:00 a. m., and 7:00 p. m., and October 22, 7:30 a. m. The calf was killed at 9:00 a. m. on Oct. 22, 1946. At postmortem examination, the lung tissue was ground in a food chopper and placed in a Baermann funnel at 1:30 p. m. Examination of a small amount of water from the funnel at 9:00 p. m. revealed several living larvae, and at 11:00 a. m., October 23, a few dead larvae were recovered. A total of 295 female and 426 male adult worms were recovered from the small intestine and 1 adult male from the abomasum.

Calf 162 was a Jersey-Angus cross born Feb. 10, 1946. A large number of infective hookworm larvae that had been cultured from calf 158 were applied to the skin of the right flank on May 11, 1946. The animal was then placed securely in a stanchion until Aug. 22, 1946. The skin of the right flank showed the usual reactions. It was itchy and small scales and scabs followed the usual exudate of lymph on the first few days following the application of larvae. On June 15, 1946, the feces had a disagreeable odor and the following day, thirty-eight days after the application of the larvae, a severe diarrhea developed (fig. 1a). The discharge contained blood, some mixed with the feces and some unmixed. This condition continued for the next eighteen days, at the end of which time the manure became more firm but intermittently was soft and had a chalky white appearance during the next twenty-eight days, after which time it became normal in appearance and consistency (fig. 1a). From June 18 to 22, the calf refused to eat the grain concentrate, either refused or drank only a portion of the regular feed of milk, and ate very little hay. Until July 15 he continued at times to refuse to eat the grain concentrate, or ate only a small part, and did not eat as much hay as usual. During this time, the calf became emaciated, and his coat became rough. By August 1, however, he began to show improvement in appetite and general condition which continued until the end of the experiment. Fecal examinations were made at regular intervals throughout the experiment (fig. 1a), and a total of 122 were consistently negative for hookworm eggs even though the animal showed all the typical symptoms appearing in other animals in this series of experiments.

Calf 163 was a grade Jersey male born on March 20, 1946. He was fastened securely in a stanchion October 10 and received applications of larvae on the left flank on that day. On October 11, applications of larvae were made on the right flank. By the following day, the areas of skin to which the larvae had been applied were itchy and by the third day the animal was beginning to show evidence of considerable irritation in the inoculated areas. By the sixth day he was so agitated that he was unable to eat hay and grain without interruption. He would lick the hayrack and the sides of the stanchion and kick forward with either hind foot in an attempt to relieve the irritation of the affected areas. This continued for the next sixteen days. At the end of this time, the skin was becoming scaly and the hair began to come out in some small areas. Between Nov. 4 and Nov. 22, 1946, the animal had a severe diarrhea and the feces had a disagreeable odor characteristic of animals in the acute stages of infection. Blood was absent from the samples, and the severity varied widely from day to day. With the exception of the typical diarrhea and the skin irritation, the calf showed no other evidence of inoculation. A total of 104 fecal examinations, 22 previous to inoculation, were made on this animal between May 14, 1946, and June 4, 1947, and all remained consistently negative to all parasite eggs, except Strongyloides.

This calf was weighed at approximately weekly intervals, and the weights are shown graphically (fig. 1a). It will be noted that he made a substantial gain during the two weeks following inoculation but during the period of diarrhea there was a gradual decline. Following the period of diarrhea, there was a steady gain in weight throughout the remainder of the experiment. This animal is of special interest along with calf 162, in that he showed all the typical symptoms of infection such as skin irritation, diarrhea, and loss in weight, but the fecal examinations failed to show the presence of any eggs.

Calf 165 was a purebred Holstein-Friesian male, born Nov. 14, 1946. On Nov. 22, 1946, infective larvae cultured from calf 159 were placed on his back. Since no stanchion was available, this animal was not confined as were the others, but the larvae were placed on his back toward the shoulders where they would be

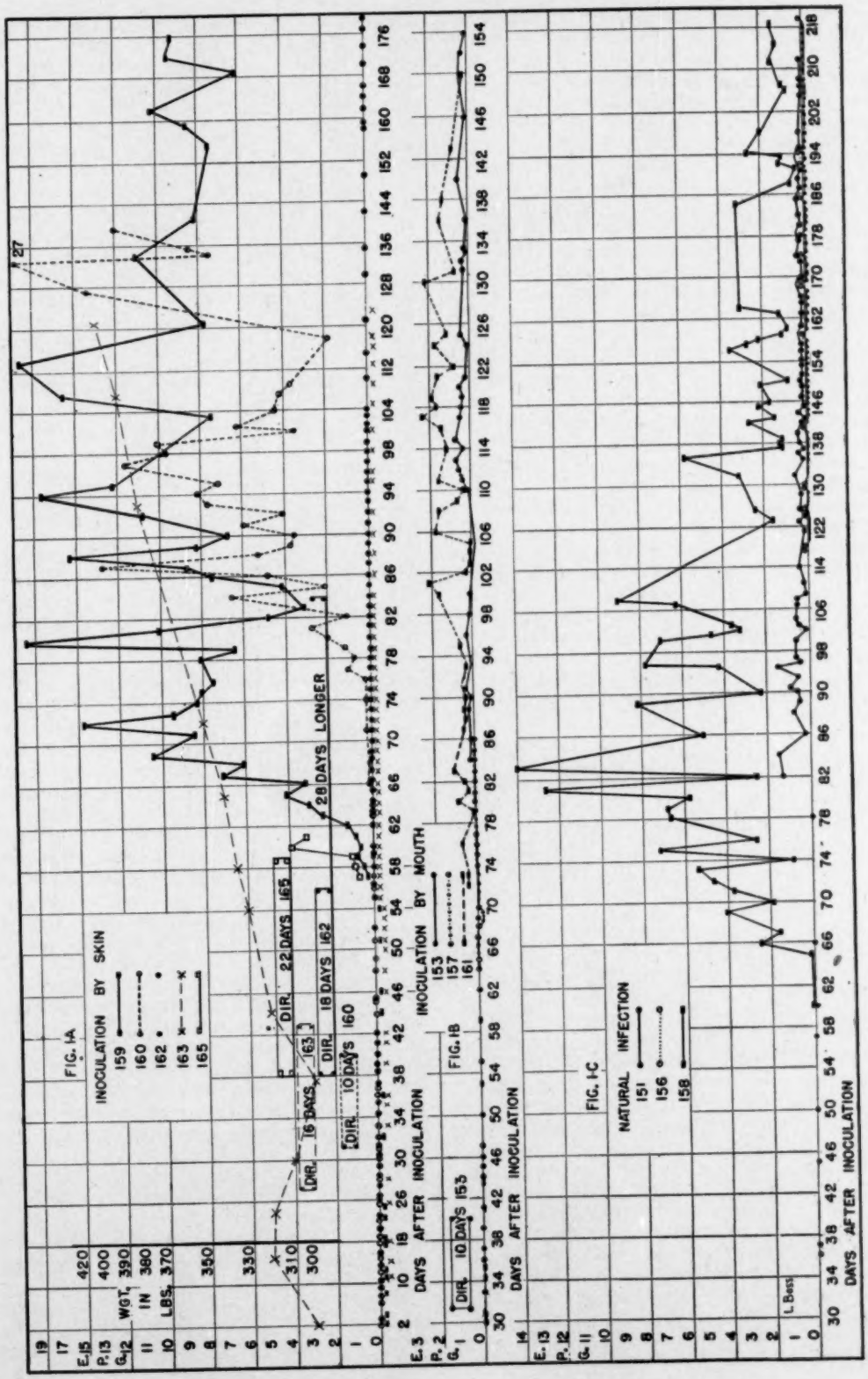
FIG. IA
INOCULATION BY SKIN
159
160
162
163
165
WGT. IN LBS.
DIR. 16 DAYS 163
DIR. 22 DAYS 165
DIR. 18 DAYS 162
28 DAYS LONGER
DIR. 10 DAYS 160
DAYS AFTER INOCULATION
FIG. IB
INOCULATION BY MOUTH
153
157
161
DIR. 10 DAYS 153
DAYS AFTER INOCULATION
FIG. IC
NATURAL INFECTION
151
156
158
L. Bess
DAYS AFTER INOCULATION

less easily reached and licked from the hair. Within twenty to thirty minutes he appeared restless and switched his tail; by the following day, the inoculation area was moist with serum. The manure remained normal until December 29, when a chalky white diarrhea appeared and was seen intermittently until Jan. 19, 1947, the thirty-eighth day after inoculation (fig. 1a). From December 30 until his death, he refused to drink any of the regular feed of milk. On January 20 he was unable to get up without assistance, and on the following morning he was unable to stand after being assisted to his feet, and drank only about half of the regular feed of milk. At 9:30 p. m., January 21, he refused to move when disturbed and on the morning of January 22 was dead.

At the postmortem examination, the abomasum was normal in appearance and free of parasites. The anterior 31 ft. of the small intestine had numerous pinpoint and pinhead petechiae in the mucosa. The wall was about normal in thickness, and a total of 635 hookworms were recovered from the apparently normal contents. The middle 22 ft. of the small intestine had the same general appearance as the anterior portion, and 158 hookworms were recovered from the contents. The wall of the posterior 30 ft. was much thickened and edematous in comparison with the anterior portion, petechiae were much more numerous, and there were some elevated pinpoint, whitish spots. The contents contained some blood, much mucus and clotted blood throughout the entire portion; 1,178 hookworms were recovered. The weight of calf 165 was 108¼ lb. at the time of his death while calf 164, another Holstein-Friesian born three days earlier, weighed 157¾ lb. Although only the one weight was taken, it appeared that calf 165 lost condition and weight rapidly during the period of diarrhea.

Inoculation by Mouth.—Calf 153 was a purebred Holstein-Friesian male born Feb. 11, 1945. He received hookworm larvae by mouth on March 13 and 21, 1946. The first hookworm eggs were recovered from the feces on June 5, 1946 (fig. 1b), which was the eighty-fourth day after inoculation. No fecal examinations were made between May 21 and June 5; however, a total of 138 examinations made previous to May 21 were consistently negative for hookworm eggs and remained negative up, at least, to the sixty-ninth day after inoculation. This calf had previously been used in a stomach worm experiment and had been consistently positive for stomach worm eggs since Nov. 12, 1945. It will be noted that the hookworm egg counts remained consistently low (fig. 1b) in all of the 45 fecal examinations made during the period of this experiment. This calf developed a mild diarrhea lasting from the thirty-second to the forty-second day after receiving the hookworm larvae. The animal was killed on Oct. 8, 1946, and 4 female and 1 male hookworm were recovered from the abomasum and 3 females and 2 males from the small intestine.

Calf 157 was a purebred Holstein-Friesian male born Sept. 28, 1945. He was given hookworm larvae by mouth on April 5, 1946, and the first eggs appeared in the feces on June 7, 1946, sixty-four days following inoculation. No fecal examinations were made between May 21 and June 7 (fig. 1b). It will be noted that the egg counts remained low (there never were more than 4 eggs recovered on any examination) in all of the 19 examinations made during the period of observation. Five of the fecal examinations were negative.

Calf 161 was a purebred Jersey male born Oct. 5, 1945. He was given larvae by mouth on Dec. 18, 22, 30, 1945, and on Jan. 30, 1946. The first eggs were recovered on Feb. 27, 1946, on the seventy-second day after receiving the first dose of larvae (fig. 2b). No fecal examinations were made between February 14 and 27, and a total of 31 fecal examinations made previous to February 27 were consistently negative. It will be noted that the egg counts were low thoughout the experiment, no counts having been obtained higher than 2 and a fraction eggs per gram of sediment.

The calf was given an application of larvae on the skin of the flank on Dec. 3, 1946, and was slaughtered on December 9, in order to check the results of the experiment and to determine if larvae could be recovered from the lungs. At postmortem examination, no larvae were recovered from the digestive tract. Ten adult females and 3 adult males were recovered from the small intestine. The lungs were ground in a meat chopper and placed in Baermann funnels at 11:00 a. m. At 1:15 p. m. and 8:00 p. m., larvae were recovered from the funnels. Larvae were also recovered from the funnels at 9:00 a. m. the following morning.

Natural Infections.—Three calves, obtained when less than 24 hours old, began passing typical hookworm eggs after they were 60 days old. These calves were kept in our animal building under our regular system of management. Since none of our animals previous to this time indicated infection with any parasite except Strongyloides sp., we were naturally concerned and thought the infection was being carried into the quarters in some manner. However, after taking all possible care and stressing all precautions, and since data has accumulated from the experimental inoculations with the hookworm, we now believe these animals secured their infective larvae in the short period between birth and removal to the experimental conditions.

Calf 151 was born on Nov. 11, 1944, and hookworm eggs were recovered from the feces on Feb. 1, 1945, when the calf was 82 days old (fig. 1c). Unfortunately, only one fecal examination was made previous to February 1, but on January 10, at the age of 60 days, no parasite eggs were recovered. The number of eggs always remained low, and no symptoms of parasitism were observed.

Calf 156, a purebred Jersey male, was born April 8, 1945. Eggs were first recovered on Aug. 1, 1945 (fig. 2c). No fecal examinations were made between June 25 and August 1; seven examinations made before June 25 at the age of 78 days were all negative, so it is evident that animals may remain negative as long as seventy-eight days after infection. This calf was slaughtered on Feb. 28, 1946, and 1 female hookworm was recovered. All of the 15 fecal examinations made after August 1 were positive but the counts were low.

Calf 158 was a purebred Holstein-Friesian male born Sept. 29, 1945. Hookworm eggs were first recovered on Nov. 28, 1945 (fig. 1c), when the calf was 60 days old. Three previous fecal examinations were negative, but none were made between November 13 and 28. The egg count reached the maximum on the eighty-third days after birth when it was 14 eggs per gram of sediment. No diarrhea or other symptoms of parasitism were observed.

SUMMARY

Inoculation experiments were carried out under controlled conditions of infection in which 5 calves became infected by placing hookworm larvae on the skin. Four of these were placed in stanchions in such a manner that they could not lick any portion of the body. Three animals were inoculated by mouth, and 3 are described which are believed to be natural infections.

Diarrhea of varying degrees of severity developed in 4 of those infected by skin and in 1 infected by mouth. The periods of diarrhea lasted from ten to twenty-two days and began from the twenty-seventh to the thirty-eighth day after inoculation. In 1 animal, there was a period of intermittent diarrhea lasting twenty-eight days following a period of eighteen days of the severe type.

One calf died on the sixty-first day following inoculation, eggs having been found only on five days preceding death and following a period of diarrhea lasting twenty-two days.

Eggs were recovered from the feces as early as the fifty-seventh day.

Larvae were recovered from the lungs of 2 calves that were slaughtered following inoculation by placing larvae on the skin.

All the symptoms observed occurred during the prepatent period of the infection. A general improvement was observed following maturing of the larvae into adults.

The above observations emphasize the importance of barn and shade sanitation in controlling hookworm infection in calves.

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Research on Swine Brucellosis by the Bureau of Animal Industry (1941-1947)

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BRUCELLOSIS in swine has been recognized as a specific disease since 1914 when Traum isolated the causative agent, *Brucella suis*. Following his discovery, this disease has been studied at irregular intervals by numerous investigators in the United States and Denmark. The degree of spread in the last thirty years and the actual incidence of the disease today are unknown, but from available evidence it is believed to be present in most swine raising areas of this country. The insidious nature of swine brucellosis has created the tendency to underestimate its economic importance to the swine industry and its potentiality as a reservoir of infection for human beings and other species of animals. We are now aware that the disease may cause severe financial loss in individual herds.

An accepted husbandry practice in many sections is to allow swine, cattle, and other Brucella-susceptible animals to occupy a common environment. It is doubtful if brucellosis can be controlled and eradicated in any one species of animals without giving due consideration to all susceptible species. This is evidenced by the work of Jordan and coworkers who reported *Br. suis* as being responsible for a milkborne outbreak of brucellosis in human beings in Iowa in 1942.

The investigations on brucellosis in swine to be discussed are methods and results of exposure, susceptibility, symptoms and lesions, localization of infection, channels of elimination, persistence of infection, diagnostic tests, and resistance.

METHODS AND RESULTS OF EXPOSURE

Brucella suis infection has been readily produced in swine by exposure through the alimentary canal, the conjunctiva, the copulatory organs, and by intradermal inoculation. Our experience has been that greater numbers of *Br. suis* of comparable virulence are required to infect swine through the alimentary and genital tracts than by the conjunctival or intradermal route.

Infection has been demonstrated in suckling pigs exposed to milk from infected dams and to contaminated premises. A very significant method of producing infection in swine and not in cattle is the transmission of the disease from male to female and vice versa when breeding. A group of 7 susceptible, sexually mature boars was exposed to sows which were persistently eliminating *Br. suis* from the vagina. Genital infection was definitely established in 3 of 5 boars exposed from breeding only, and in the 2 other boars from teasing only. In an attempt to prevent any chance for exposure through the mouth in the breeding boars, the sows were thoroughly washed after the teasing procedure and placed in clean quarters for mating. Two groups containing 8 and 6 sows each were bred to boars secreting *Br. suis* in the semen, and infection was demonstrated in all but 1 animal. One group of 8 sows and two groups of 9 each were exposed through the mouth, the conjunctiva, and by intradermal inoculation, respectively. Brucella recoveries were made from all the sows in each group.

Because of the massive numbers of organisms that have been observed in the body secretions and excretions of swine, the probability is that the spread of the disease occurs as frequently at the time of breeding as in feeding.

DISCUSSION

Susceptibility.—Swine of both sexes and all ages are susceptible to *Br. suis*. The rate and character of the infection are influenced by the degree and virulence of the exposure and the susceptibility of the individual. When a known infective dose of *Br. suis* is given and evidence of the disease is based on recoveries from the blood and/or from specimens collected either at the termination of pregnancy or at the time of autopsy, the infection rate has been as high as 100 per cent in some

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groups of mature swine. However, some of the swine which show a bacteriemia for only a short time do not become permanently infected as indicated by the failure to recover Brucella at the time of parturition or autopsy. Other exposed swine may only develop a low transient titer. In pregnant sows, the period of gestation appears to have no influence on susceptibility.

Studies were conducted on 230 suckling pigs that were exposed to dams secreting infected milk and to contaminated pens until weaning time. The weaning age of the pigs in the different litters varied from 58 to 84 days. Immediately after weaning they were isolated on clean ground. Blood specimens were collected for bacteriologic and serologic determinations at the time of weaning and thereafter at frequent intervals up to 3 years of age. Pigs were considered as having been infected during the suckling period if there was any evidence of blood agglutinins in the 1:25 dilution or if *Br. suis* had been isolated from the blood up to six weeks following weaning. At this time, 10 of 127 males and 17 of 103 females were considered as infected animals. After the sexes were separated, 17 more females which had not shown any evidence of infection were placed with the female group as controls. All but 1 of the controls developed low transient titers in from three to twenty-seven months following exposure to the infected group. Five additional 8-month-old gilts from a Brucella-free source were added to the group six months following weaning, and all developed low transient titers as the result of this contact exposure.

Symptoms and Gross Lesions.—The clinical manifestations of swine brucellosis may be variable and obscure. From our observations, clinical evidence of the disease is usually absent in swine infected as suckling pigs. Of the 10 boars infected in this manner, only 1 showed any evidence of the disease, and this was manifested by orchitis followed by a progressive paralysis. In a similar group of 17 infected sows, none aborted and no significant symptoms were observed.

Sterility and orchitis have been the symptoms most commonly observed in boars infected as adults. Occasionally, a boar has developed lameness or posterior paralysis. However, the absence of orchitis in infected boars is no assurance that the infection is not present elsewhere in the urogenital tract.

The symptoms that have been observed in sows infected as adults are abortion, birth of weak or stillborn pigs, metritis, sterility, lameness, and posterior paralysis. Abortions have occurred from the twenty-second to the thirtieth day of gestation. Under farm conditions, these early abortions may pass unobserved, thus creating the impression that the sow had failed to conceive. The other extreme has been the farrowing of full-term litters associated with uterine infection. Sows with persistent uterine infection usually have an irregular estrual cycle and fail to conceive after numerous matings to boars giving negative reactions to the test. Only 1 sow with chronic uterine infection has had a noticeable discharge. However, at the time of autopsy, a thin puslike exudate accompanied by marked inflammation of the uterine mucosa was observed in 3 sows so far examined.

Lameness and paralysis have occurred more frequently in some groups of infected swine than in others and should not be overlooked when brucellosis is suspected. When paralysis is due to spondylitis resulting from Brucella infection, the gross lesions most often observed appear as necrosis of the intervertebral cartilage and bodies of the adjacent vertebrae. The lesions in the vertebrae have the appearance of progressive necrosis from the periphery to the center of the bone structure.

Localization of Infection.—Brucella suis may localize in most of the body tissues of swine. The tissues routinely collected for culture purposes were the spleen, liver, all urogenital organs, seven groups of lymph glands from various regions of the body, and affected joints and areas of the spinal column. Bacteriologic studies have been completed on 250 experimental swine at the time of autopsy. Of this group, 165 sows and 17 boars had been artificially ex-

posed, and 54 sows and 14 boars had received exposure by contact with infected animals.

In the 10 boars infected as suckling pigs, *Br. suis* was isolated from the urogenital organs of 1 killed at 16 months of age and from the semen of another when 3 years old. In the group of 17 sows infected in the same manner, Brucella isolations were made from the lymph glands of 2 which had died at 6 months of age. Twelve more were destroyed between the ages of 24 and 36 months, and all cultural results were negative. However, *Br. suis* was recovered from lymph glands of 3 of 16 original controls killed at the same age as the principals. It has not been determined if the infection was carried over from the suckling period or was the result of contact exposure. When future breeding stock is selected from the progeny of infected swine, our findings emphasize the necessity for testing pigs from weaning until breeding time, with the prompt removal of all reactors.

In summarizing the bacteriologic studies on the tissues of boars, localized infection was demonstrated in 28.5 per cent of the naturally exposed group and in 88 per cent of those exposed artificially. The shorter period between exposure and autopsy and the amount of exposure in the artificially exposed boars are suggested as reasons for the discrepancy in rate of recovery between the two groups. The affinity of this organism for the genital tract of boars is demonstrated by the recovery of *Br. suis* from these organs in 94.6 per cent of the infected animals. Points of localized infection in the reproductive tract are variable. *Brucella suis* has been isolated from the semen samples of some boars in which no testicular involvement could be demonstrated at autopsy. In view of these findings, it is apparent that the boar is a significant source of exposure.

In sows, *Br. suis* was recovered from 18.1 per cent of the artificially exposed animals as compared with 24 per cent that were naturally exposed. The most common sites of localized infection were the lymph glands. From the infected animals, the percentage and order of isolations were 77.3 from lymph glands of the head, 68.1 from lymph glands of the viscera and body, 29.5 from the spleen, 23.2 from the vertebral column, 18.6 from the urogenital organs, and 18.6 from the liver.

When swine were slaughtered and cultured nineteen days following exposure, *Br. suis* was isolated from the blood, muscles, lymph glands, and numerous organs.

From the autopsy findings, indications are that the disease is self-limiting in approximately 75 per cent of the swine. This figure will no doubt fluctuate in different herds, depending on the virulence of the infection and susceptibility of the swine.

Channels of Elimination and Persistence of Infection.—Studies on the channels of elimination and the persistence of dissemination in both boars and sows have demonstrated that *Br. suis* may be eliminated in the urine, semen, vaginal discharge, and milk. Urogenital, udder, and blood infections have been found to persist for much longer periods than had been previously established. Our results indicate that a higher percentage of boars than sows eliminate *Br. suis* from the genital tract. Also the organism has been isolated more frequently from the urinary bladder of boars at the time of autopsy. *Brucella suis* was consistently recovered from the semen of a 3-year-old boar that had been infected as a suckling pig. A bacteriemia was demonstrated in this animal up to 10 months of age. Active infection was produced in 4 of 5 sows bred to this boar. In the majority of boars that have persistently secreted infected semen, *Br. suis* has been isolated from the seminal vesicles at the time of autopsy. It is unusual to make recoveries from the semen of many boars at each ejaculation. From the information available, it has been impossible to correlate this phenomenon with the site of localization in the genital tract. The infective organism has been isolated from the semen and blood of boars for as long as five months following artificial exposure.

In a group of 40 sows, the length of time that *Br. suis* was eliminated was determined in 17 which had been exposed

through the conjunctiva during pregnancy and in 23 others exposed by the vagina when bred. Urine, vaginal swabs, and blood were collected for routine laboratory examination at weekly or biweekly intervals. Fifty-eight per cent of the eye-exposure group had a urogenital infection which varied in duration from one to thirty-one months. The average duration of *Br. suis* recoveries from the blood was eight and three-tenths (8.3) months, with the longest time being thirty-four months. In the group exposed by the vagina, 47.8 per cent had a urogenital infection that persisted from three to thirty-one months. The average duration of blood stream infection was eight and six-tenths (8.6) months, the most persistent being twenty months. Positive Brucella recoveries from the vagina or urine were not necessarily associated with a vaginal discharge or any particular phase of the estrual cycle. In a sow bred to an infected boar, *Br. suis* was regularly isolated from the vagina for three months prior to farrowing, at termination of a normal gestation, and for three subsequent months. At farrowing time, numerous Brucella were isolated from the uterine discharge, but upon autopsy the 11 vigorous and 1 stillborn pigs were free from infection.

A total of 18 sows which had udder infection at termination of the first gestation were rebred after a rest period of approximately seven months. Only 2 had a chronic udder infection at the time of their second farrowing. The number of animals employed is limited and may be insufficient to warrant an accurate estimate on the expectancy of chronic infection in the udder. However, *Br. suis* has not been isolated from the nonfunctioning udder tissue of any of the sows at the time of autopsy. If these findings are representative of the disease in swine, the seriousness of urogenital infection in both sexes must not be overlooked in the control of the disease.

Diagnostic Methods.—The present diagnostic methods for the detection of brucellosis in swine are inadequate for adopting comprehensive control measures. Three limiting factors which make the agglutination test unreliable for detecting infected individuals are (1) the absence of agglutinins in the blood of swine in the early stages of the disease; (2) the presence of low transient blood titers in some infected animals; and (3) the recedence of blood titers in a significant number of others.

Recoveries of *Br. suis* from the blood of swine prior to the development of agglutination titers have not been unusual. Brucella isolations have also been made from the semen of 1 boar and the vaginal discharge of 4 sows after the concentration of blood agglutinins had receded to the extent that detection was impossible in the 1:25 dilution.

However, it has been our experience that the majority of infected swine will develop a blood titer of 1:100 or higher for at least a short period following exposure. A sufficient number have retained this blood titer to make the agglutination test applicable for the diagnosis of swine brucellosis on a herd basis. Consequently, all control measures must be applied to the herd instead of to the individual.

Both standard *Brucella abortus* plate and tube antigen have been employed for blood titer determination, and no appreciable discrepancies have been noted between the two. All swine serum samples were routinely tested in the 1:25 and higher dilutions. Limited studies on modified antigens prepared from Br. *suis* have resulted in no ap preciable improvement over the standard *Br. abortus* antigen as a diagnostic agent.

Comparative blood serum agglutination and allergic tests have been completed on 111 swine previously exposed to virulent *Br. suis* by natural and artificial methods. These tests were administered approximately two weeks prior to autopsy and bacteriologic examination of tissues. The diagnostic agents employed were standard *Br. abortus* antigen and Brucellergen prepared from *Br. suis* by Huddleson. *Br. suis* was recovered from the tissues of 24 swine at the time of autopsy. With a blood titer of 1:25 as evidence of infection, the agglutination test identified 83.3 per cent and the allergic test 91.6 per cent. Of the remaining 87 swine from which no infective or-

ganisms were recovered, 69 per cent reacted to the agglutination test as compared with 86 per cent which reacted to Brucellergen. The high percentage of reactions to both tests in swine that have apparently recovered may be the result of sensitization produced by exposure to *Br. suis*, or some animals may have been harboring the infection in such small amounts that recovery was impossible with cultural procedures employed.

Resistance.—Studies on resistance were conducted with *Br. abortus* strain 19 and a *Br. suis* strain identified as King No. 8.

Twenty gilts between 4 and 5 months old were inoculated subcutaneously with strain 19; 10 received 5 cc. and the other 10 received 10 cc. The vaccine had a concentration of 20 billion viable Brucella per cubic centimeter. The average maximum postvaccinal titer was 1:300 at eight days. The average agglutination reaction six months following vaccination was positive in a dilution of 1:12.5. Resistance was not challenged in 5 of 6 sows that proved to be nonbreeders. The remaining sows, including 1 which was not pregnant, were exposed to virulent *Br. suis* during the middle one-third of pregnancy. Exposure took place six months following vaccination. Two open and 9 pregnant control sows were exposed in the same manner as the vaccinated animals. Resistance is based on bacteriologic findings in blood cultures, colostrum, uterine material, fetuses, and body tissues. The infection rate in the vaccinated sows was 80 per cent as compared with 81.8 per cent in the controls. However, positive Brucella recoveries from the blood cannot be correlated with the course of the disease nor are they an indication that the infection will become localized. Many exposed sows will have a bacteriemia for two or three weeks and apparently recover, as indicated by bacteriologic findings at the time of parturition and autopsy. If the infection rate is based on Brucella recoveries at the time of parturition and autopsy, then 66.6 per cent of the principals and 45.4 per cent of the controls were infected. From these results, it would appear that strain 19 has little or no value as an immunizing agent against virulent *Br. suis* infection.

Similar vaccination experiments were carried out on swine with a *Br. suis* culture (King No. 8) of reduced virulence as the vaccinal agent. It should be emphasized at this time that although the vaccine strain is of reduced virulence, too little is known about its stability to warrant its use. In the first of a series of 24 gilts, 12 were injected subcutaneously with 5 cc. and 12 with 10 cc. The vaccine had a concentration of 20 billion viable cells per cubic centimeter. The average postvaccinal titer was 1 : 400 at thirteen days. Nine months following vaccination and at the time of exposure, the average titer was positive 1 : 12.5. Twenty-four control sows and 24 vaccinated sows of the same age were exposed to virulent *Br. suis* in the same manner as the strain 19 vaccinated group. One of the principals and 3 controls were not pregnant. Virulent *Br. suis* was recovered from 58.4 per cent of the vaccinated sows as compared with 87.5 per cent of the controls. Postexposure hemoculture studies showed that the degree and persistence of agglutination titers and blood stream infection were considerably less in the vaccinated group. Again, however, if infection is determined on the basis of recoveries at the time of parturition and autopsy, 20.8 per cent of the principals were infected as compared with 66.6 per cent of the controls. The size of the two doses received at vaccination had no influence on the degree of resistance induced.

A second series of 30 gilts, 6 months old, were vaccinated with the *Br. suis* (King No. 8) vaccine to further determine the degree of virulence of this strain and to ascertain the duration of resistance induced. The concentration of vaccine used was 10 billion viable bacteria per cubic centimeter. Twenty animals were inoculated with 10 cc. and 5 with 5 cc. of vaccine subcutaneously, and the remaining 5 were injected intracutaneously, 2 with 0.2 cc. and 3 with 1 cc. Blood specimens were collected at weekly intervals following vaccination for culture and titer determinations. The vaccine strain was recovered but once from a gilt vaccinated with 0.2 cc. by the intradermal route. The average maximum

postvaccinal agglutination titer was 1 : 800 on the seventh day and receded to 1 : 12.5 by nine months. The resistance was challenged in 27 vaccinated and 15 control sows at twenty-four months. *Brucella suis* was isolated from 96 per cent of the principals and 100 per cent of the controls. These findings indicate that although varying degrees of resistance are induced in swine by exposure to *Br. suis*, the duration of this protection is for a relatively short time.

SUMMARY

1) *Brucella suis* infection can be readily produced by exposing swine through the alimentary canal, the copulatory organs, conjunctiva, and by intradermal inoculation.

2) Swine of both sexes and all ages are susceptible to *Br. suis*.

3) The clinical manifestations of swine brucellosis vary in character and intensity. Usually one or more symptoms, such as abortion, weak or stillborn pigs, sterility, orchitis, paralysis, and lameness, are observed in an infected herd.

4) *Brucella suis* may localize in most of the body tissues. Infection has been demonstrated more frequently in the reproductive organs of boars than of sows.

5) Urogenital infection may become chronic in both boars and sows with persistent elimination of the infective agent. *Brucella suis* has been isolated from the semen of a 3-year-old boar infected as a suckling pig and from the vaginas of sows for as long as thirty-one months.

6) Persistent blood infection has been demonstrated for thirty-four months in swine.

7) Both the standard plate and tube agglutination tests are inadequate for the diagnosis of brucellosis in individual swine but have been effective in diagnosing herd infection. The diagnostic agent, Brucellergen, does not appear to be any more reliable than the agglutination test for the diagnosis of brucellosis in the individual.

8) Vaccination of 4- to 5-month-old gilts with *Brucella abortus* strain 19 failed to produce any detectable immunity for six months.

9) Vaccination of 4- to 6-month-old gilts with a *Br. suis* strain of reduced virulence produced a serviceable immunity for nine months but was ineffective after twenty-four months. Although the vaccinal strain was of reduced virulence, too little is known about its stability to warrant its use.

DISCUSSION

DR. CLARENCE M. HARING (Berkeley, Calif.).—I would like to ask if immunity results.

DR. MANTHEI.—We exposed about 20 animals, and in this group we recovered Brucella from 2 at the time of parturition, and we did not recover the organism from the blood at any time following the second exposure. However, Dr. Hutchings is in the room. They have done some work at Purdue on this same thing, especially on animals infested as suckling pigs. They have been able to infect swine by reëxposure. Dr. Hutchings, would you care to comment on that?

DR. L. M. HUTCHINGS (Lafayette, Ind.).—We had 13 out of 19 which gave definite bacteriologic evidence and became reinfected. Abortions, however, were definitely miniminzed; only one abortion out of 19.

In 11 naturally infected sows which we acquired, 5 gave positive bacteriologic results upon reëxposure through naturally infected material. Five were still sterile, and we couldn't produce pregnancies in them. Those have been the essential results.

The Interpretation of Low Agglutination Titers in the Control of Swine Brucellosis

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SINCE the symptoms of brucellosis in swine are rather inconstant and indefinite, the detection and diagnosis of the disease depend upon bacteriologic and serologic techniques. Inasmuch as bacteriologic techniques involve certain laboratory procedures not usually available under general field conditions, this method of diagnosis is limited in its practical application. Serologic techniques on the other hand, especially the agglutination test, have proved valuable diagnostic aids when properly performed and interpreted. The diagnostic value of the agglutination test is greatest when it can be applied to all the swine in the herd and least valuable in individual animals. This paper aims to point out some of the factors to be considered when interpreting the results of tests in herds that are apparently free from brucellosis but in which an appreciable number of the animals show titers in the lower dilutions (1:25 to 1:50).

The interpretation of low titer reactions in swine brucellosis was the objective of a study by Cameron.[1] Twenty-one herds containing 933 swine were studied. Tests on 226 animals in 11 herds believed to be free from Brucella infection showed a complete absence of agglutination in dilutions of 1:25 and higher, whereas 65 per cent of 327 animals tested in ten infected herds showed complete agglutination in the 1:25 or higher dilutions. This strongly suggests, according to Cameron, that a reaction in the 1:25 dilution is specific and should be regarded as evidence of exposure to Brucella.

In this connection, Cameron, Gregory, and Hughes,[2] and also Hutchings[3] report the isolation of *Brucella suis* from the blood stream and/or tissues of swine that were either completely negative agglutinogenically or were carrying titers of 1:25 or 1:50.

It is important to remember that some Brucella-infected animals may not show agglutinins in their blood serum, or they may be present in low concentration. It is also important to know that positive or complete agglutination in the lower dilutions sometimes occurs in swine that are free from the disease. For the past several years, we have been particularly interested in the agglutination titer profiles in herds which, for various reasons, are believed to be free from brucellosis. The titer profile is the percentage of the animals in the herd that shows agglutination in the various dilutions.

EXPERIMENTAL

In 1936, a brucellosis testing program was begun in one of the herds of swine under our supervision for disease control. It has been continued each year. Some years as many as 38 per cent of the pigs, 9 or more months of age, showed incomplete or complete agglutination largely in the 1:25 dilution and a few in the 1:50. Other years the number was as low as 14 per cent. Similar results have been obtained in other herds, some of which have been tested annually for six years, others for five years, and still others for four, three, and two years. In eight herds, only one test on all of the swine of breeding age has been conducted thus far. In these herds, which appear to be free from brucellosis, it has been the rule, rather than the exception, to find titers in the 1:25 and 1:50 range. The difference between these positive findings and the negative ones reported by Cameron may be the differences in the antigens used in conducting the agglutination test. Cameron did not state what antigen was used in his studies. Therefore, it is impossible to make a comparison between his results and those reported in this study.

The tube method of conducting the agglutination test was employed. It was set up to include dilutions of 1:25, 1:50, 1:100, and

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1:200. Standard *Brucella abortus* antigen, furnished by the United States Bureau of Animal Industry for the testing of cattle in the bovine brucellosis control program was used in the major portion of the tests. The antigen used had a density of 0.04 per cent bacteria by volume (or a density approximating McFarland 1 to 1.5 or Gates 6 to 8). The test mixtures were incubated at 37.5 C. for forty-eight hours before reading the agglutination titers.

The swine in five of the herds mentioned previously constitute the subjects of this report. These were selected because detailed information of the reproductive history on each of the animals was available. This we construe to be an important part of the study. The data cover a period of four consecutive years (1943 to 1946) on four herds, and two consecutive years (1945 and 1946) on the fifth herd. The youngest animals were approximately 7 months of age when tested and the oldest was 53 months. Of the females, 65.3 per cent were gilts and were bred to produce their first litter, 24.0 per cent were sows bred to produce their second litter, 9.2 per cent to produce their third litter, and 1.5 per cent to produce a fourth litter. With respect to the males, 69.0 per cent were used as sires for one breeding season, 30.0 per cent for two breeding seasons, and 1.8 per cent for three seasons. Tests were also made on other swine in these herds but, since they were not retained in the herd for breeding purposes and because no specific reproductive information was available, they are not included in the data. The testing was done only once each year in October or November. With few exceptions, the testing was completed before the breeding season began.

For the purpose of this report and for the sake of simplification, incomplete or complete agglutination reactions in a particular dilution are called positive reactions. This reduces the number of titer categories (table 1) for the purposes of comparison and interpretation.

When a mating was arranged, no consideration was given to the results of the agglutination test on either the male or the female. Thus, males that were negative to the test in all dilutions were mated with females that were likewise negative in all dilutions and/or with reacting females. Table 1 shows the mating combinations that resulted when arranged on the basis of the agglutination test results and also the number of times each combination occurred.

Of the litters, 349 (54.0%) were the progeny of sires and dams that were negative to the agglutination test in all dilutions before breeding. In 121 (18.7%), the sires were negative in all dilutions, but the dams carried agglutination titers of either 1:25 or 1:50. The sires of 145 (22.4%) of the litters were positive in the 1:25 dilution only. They were mated with 82 females that were negative in all dilutions, 59 that were positive at 1:25 only, and 4 that were positive at 1:50. The sires of 32 (4.9%) of the litters carried maximum titers of 1:50. Of the dams mated with them, 14 were negative in all dilutions and 18 carried titers of 1:25.

Table 1 also shows the reproductive performance of the dams with respect to the average number of pigs per litter born living, the average number per litter born dead, and the average number weaned per litter at 56 days of age. The results suggest that there are no important differences in the number born living and the number born dead when both parents are negative to the agglutination test in all dilutions, or when 1 parent is negative and the other parent positive in one or both serum-antigen dilutions, or when both parents are positive. The grand averages for pigs born living and pigs born dead per litter are 7.85 and 0.44, respectively. The loss which occurred between the time of birth and the time of weaning equals 28.8 per cent. This latter figure is quite characteristic of the mortality statistics that cover the nursing period (56 days, or 8 weeks) as generally reported. Some of the pigs that were weak at birth and died within the first week of life were examined bacteriologically, but no organisms of the Brucella species were recovered. Losses from an acute "toxemia" syndrome oc-

TABLE 1—Mating Combinations in Relation to Agglutination Reactions and Reproductive Performance in Swine

Agglutination reactions			Agglutination reactions			No. of litters produced	Av. no. pigs born per litter		Av. no. pigs weaned per litter	Weaned (%)
Males			Females							
1/25	1/50	1/100	1/25	1/50	1/100		Living	Dead		
—	—	—	—	—	—	349	7.79	0.51	5.64	72
—	—	—	+	—	—	111	7.90	0.48	5.21	66
—	—	—	+	+	—	10	7.60	0.20	5.30	70
+	—	—	—	—	—	82	8.25	0.31	5.97	72
+	—	—	+	—	—	59	7.74	0.28	4.98	64
+	—	—	+	+	—	4	9.00	0.25	7.00	78
+	+	—	—	—	—	14	7.71	0.35	6.50	84
+	+	—	+	—	—	18	7.33	0.22	6.16	84
+	+	—	+	+	—	0	0.00	0.00	0.00	..

curred in one of the herds at each of two successive spring farrowing seasons.

During the period covered in this report (1943 to 1946, inclusive), 2 pigs in one of the herds and 1 pig in another herd showed agglutination in the 1:100 dilution. The 2, a boar 18 months of age and a gilt 7 months of age, were members of a herd, the titer profile of which read: 74.6 per cent negative in all dilutions, 21.3 per cent positive in 1:25, 1.3 per cent positive in 1:50, and 2.6 per cent positive in 1:100. The pig with the 1:100 titer in the second herd was a 7-month-old gilt. The titer profile on this herd was: 60.7 per cent negative in all dilutions, 32.7 per cent positive in 1:25, 5.5 per cent positive in 1:50, and 0.9 per cent positive in 1:100. Although none of the 3 animals showing reactions in the 1:100 dilution was believed to be infected with Brucella, nevertheless, they were removed from the breeding herd since this appeared to be the more conservative course to pursue.

It was stated earlier that the five herds constituting the basis of this report are considered to be free from brucellosis. There is nothing in the histories of these herds which, from the standpoint of symptoms such as abortion, sterility, orchitis, and epididymitis, would suggest the presence of the disease. From the standpoint of the agglutination test results, testing from 80 to 120 in each herd annually, all but the 3 animals referred to failed to show agglutinins in dilutions above 1:50. In fact, a majority (73.3%) of all the animals tested were negative to the test in all dilutions, 23.5 per cent were positive in the 1:25 dilution only, and 3.2 per cent positive in the 1:50 dilution. Our experience in Brucella-infected herds has been that with few exceptions the agglutination titers are much higher. In such herds, a complete agglutination in the 1:400 dilution is very common and, not infrequently, agglutination occurs in the 1:800 and 1:1,600 dilutions. In our experience, titer profiles in infected herds are of the order: 25 per cent negative in all dilutions, 25 per cent positive in 1:25, 15 per cent positive in 1:50, 15 per cent positive in 1:100, 20 per cent positive in 1:200 or higher. Cameron[1] found 34.2 per cent negative, 8.2 per cent positive in 1:25, 11 per cent positive in 1:50, 6.4 per cent positive in 1:100, 40 per cent positive in 1:200.

The reproductive performance of the sows and the livability of their progeny for the herds included in this report conform to a pattern which is characteristic of the birth and death experience for newborn and nursing pigs in general. The percentage born living was 94.6 and the percentage born dead was 5.6. We have no satisfactory records on the livability of pigs born to a larger number of "reactor" sows.

RESULTS

The results of this study demonstrate the value of a full and complete history with respect to any obvious or even suspicious signs of Brucella infection and of the reproductive performance of the herd when it comes to the matter of interpreting the results of the agglutination test. In these five herds, the positive reactions in the 1:25 and 1:50 dilution proved to be of no significance in so far as being diagnostic and specific for brucellosis. The validity of this contention has strengthened with the passing of time. In a great many cases, the agglutination titers remained unchanged from year to year. This statement applies to many animals that were tested twice and to others that were tested three times. In some animals, the titer increased slightly from one test to the next. A recession of the titer occurred in a few instances. Where this occurred, it involved a change between the 1:50 and 1:25 dilutions more often than between 1:25 and a completely negative reaction.

The physical appearance of the agglutination reactions from the standpoint of flocculation and sedimentation of the bacterial cells was, in general, different from that usually encountered in infected herds. The aggregate of cells was smaller and more difficult to read and much more easily dispersed on shaking the tube than is usually observed in tests in reactor herds. The same is true of rapid or plate tests. However, the differences are not sufficient to be diagnostic. As the herds are considered to be free from brucellosis, the results strongly suggest that the reactions in these cases represent the presence of nonspecific substances in the blood which will agglutinate Brucella antigen. Whether they represent the so-called "normal" or "native" agglutinins or agglutinins produced by one or more different microörganisms that will cross agglutinate Brucella antigen has not been fully determined. Studies on this phase of the problem are underway at the present time. The importance of knowing that nonspecific agglutination in the lower dilutions may oc-

cur in some swine is imperative since it may interfere considerably with the interpretation of the test results in some herds.

In view of the fact that some animals in Brucella-infected herds carry infections without showing titers above 1:25 or 1:50, and in view of the fact that the majority of brucellosis-free herds in our experience contain an appreciable number of animals with titers in the 1:25 or 1:50 range, it would not seem safe to declare a herd free of brucellosis on the basis of a single herd test of all breeding stock even though no titers of 1:100 or higher are disclosed. However, with the agglutination test as we use it, our experience to date suggests that in new herds being tested the first time, if such herds pass two successive herd tests (all breeding stock) thirty to sixty days apart with no titers in the 1:100 range or higher they may, with reasonable safety, be considered brucellosis-free, providing there were no additions of animals of unknown status to the herd within thirty days preceding the first herd test plus an otherwise negative herd history. Such herds on subsequent annual tests of all breeding stock may, with reasonable safety, be considered free of brucellosis if no reactions of 1:100 or higher are disclosed and no animals of unknown status have been added to the herd within sixty days preceding the test.

We are in full accord with the idea of testing the entire herd before rendering a final interpretation on the result of a test on one or several individuals in the herd. This applies particularly to tests that are negative in all dilutions as well as any that would be positive in 1:25 or in 1:50. New animals introduced into a brucellosis-free herd should come from herds of similar status. If the status of the herd from which such introductions are obtained is not known, then it is desirable to require that animals have a negative test (at 1:25) at the time of purchase and these be kept in strict isolation for sixty days. At this time, they should be required to pass a second negative test before they are allowed to become an integral part of the herd.

SUMMARY

Five brucellosis-free herds of swine have been studied in detail with particular reference to the reproductive performances for periods of two to four years. A significant percentage of the breeding animals in these herds showed agglutination titers to Brucella antigen in the 1:25 to 1:50 range with several manifesting incomplete agglutination at 1:100.

On the basis of the agglutination test, various mating combinations were made, such as between males negative at 1:25 and females that were either negative at 1:25, or positive up to 1:50, or between males that were positive at 1:50 and females with titers of 1:25.

The data on reproduction resulting from these different mating combinations show what is considered to be normal farrowing and livability of the progeny.

It is believed a herd can be satisfactorily diagnosed as brucellosis-free if two successive agglutination tests sixty days apart on all breeding stock do not disclose titers of 1:100 or higher, and no new animals have been introduced into the herd within thirty days preceding the first test.

Subsequent, single animal tests of all breeding stock which do not disclose titers in the 1:100 range or higher may be interpreted as representing a brucellosis-free herd if no new animals have been introduced into the herds within sixty days preceding the tests.

New animals to be added to a negative herd should be secured from a herd known to be free of brucellosis. If it is necessary to obtain additions from herds of unknown status, such additions should be required to pass a negative test at the time of purchase and kept in strict isolation for sixty days followed by a second negative test before the animals are allowed to become an integral part of the herd.

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The Etiology of Swine Dysentery

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DYSENTERY in swine was recognized at the Indiana Agricultural Experiment Station as a distinct disease nearly thirty years ago.[1] It was found that the feeding of bowel discharge or portions of the large intestine from affected hogs consistently reproduced the disease, while the feeding of small intestine and other viscera gave negative results. Early efforts at determining the specific cause of swine dysentery resulted in isolating a strain of *Salmonella choleraesuis* which caused enteritis when fed in cultures. It was apparent, however, that the disease produced by feeding cultures of Salmonella was not identical with the disease which was occurring naturally.

Early in the investigation of swine dysentery, it was noted that Vibrios were present in enormous numbers in the bowel discharge and on and in the cecal and colic mucosa of affected animals. The presence of these Vibrios would, of course, not necessarily be significant since morphologically similar microörganisms were known to occur in the intestinal tract of apparently healthy swine and in various other places.

In 1943,[2] a Vibrio was isolated in apparently pure culture from the colon mucosa of a hog infected with dysentery. At first considerable difficulty was encountered in propagating the Vibrio on artificial mediums. However, at that time some cultures were fed to hogs and some of these animals developed dysentery. More recently, the Vibrio has been propagated quite consistently by making transfers at intervals of about forty-eight hours. Blood agar (10% of defibrinated blood in tryptose agar) was found to be well suited for isolating and maintaining the microörganism. During 1946 apparently pure cultures of the Vibrio, mixed with gastric mucin, were fed to pigs. The use of gastric mucin can reasonably be considered as having a protective effect on the Vibrio comparable to the action of the mucus that occurs abundantly in the bowel discharge of affected hogs. Six culture feeding experiments were conducted with 10 pigs in each experiment. Typical dysentery developed in five of these six experiments. All of the 50 pigs in the five experiments developed dysentery. A period of twenty-seven days or more passed between the time the cultures were fed and the appearance of symptoms. Dysentery did not occur in the control animals.

The method used for isolating the Vibrio from the mucosa of the large bowel was to enter the bowel either from the serous or mucous surfaces and snip out a small piece from the depth of the mucosa. This small piece of tissue was then streaked on a series of blood agar plates. The plates were either sealed with tape or placed in sealed culture jars containing about 15 per cent of carbon dioxide. Incubation was at 37.5C. Usually about forty-eight hours were required for easily recognizable growth of the Vibrio. The first growth was scant and usually occurred as roundish, dewdrop-like colonies, but sometimes elongated colonies developed along the track where the piece of mucosa was drawn over the surface of the blood agar.

Culturally, this Vibrio resembles *Vibrio fetus* perhaps more closely than any other Vibrio which has been found associated with disease in livestock. It caused relatively little change in culture mediums commonly used. In observations made thus far it did not liquefy gelatin, reduce nitrate, or produce indole. No change was observed in litmus milk or in broth containing the

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following carbohydrates: dextrose, lactose, saccharose, maltose, and mannite. It did not cause hemolysis in blood agar plates. When fairly abundant growth occurred on blood agar, after several transfers, there was a well-marked darkening of the medium. The growth was usually abundant in the moisture of condensation. Spurs of heavy growth sometimes extended upward in the angles between the walls of the test tubes and the slanted medium.

Morphologically, the Vibrio shows considerable variation, depending upon the age and the culture medium on, or in, which it is grown. When freshly isolated, the individual cells are usually distinctly comma-shaped and possibly somewhat spiral-formed; and most of them occur singly. After several generations on artificial medium, long chains may be formed. When grown in tryptose broth, plump cells are often seen. Granule formation has been noted frequently, particularly in older cultures. Motility is well marked, propulsion is by a polar flagellum or flagella. The Vibrio stains well with dilute fuchsin and is gram-negative.

The pathogenicity of the Vibrio has been tested to a limited extent on rabbits, white rats and mice, guinea pigs, chickens, and 1 calf. Cultures of the organisms were introduced parenterally into these species except the calf. The calf and also some rabbits were exposed by being fed cultures. The results of these exposures were negative. It is suggested that this Vibrio be called *Vibrio coli.*

DISCUSSION

DR. DURANT.—Is there any relation of this Vibrio to the one commonly observed in the ulcers in chronic hog cholera? I recall many years ago making some studies of hog cholrea, and with a dark field we could demonstrate Vibrios frequently in the ulcers of the iliosacral valve.

DR. DOYLE—Ford, I think, was the first one to report the occurrence of Vibrios in the intestinal tract of apparently normal hogs. If further observations on this microörganism indicate that it is a specific cause of dysentery, and if further work is done with it, undoubtedly we will have the problem of distinguishing between it and the one that Dr. Durant has observed.

We are not sure, but we think we can see morphologic differences between this Vibrio and the one ordinarily seen in the intestinal tract of hogs with cholera.

DR. J. M. MURPHY (Ithaca, N. Y.).—Is there a satisfactory treatment?

DR. DOYLE.—I can answer briefly by saying no. We have tried quite a number of the sulfa drugs but have found none that are promising as a specific treatment. Various treatments are used and undoubtedly do some good.

Careful nursing, careful feeding, sanitation, and isolation are the only things we can recommend at present; in fact, we usually recommend that the farmer go out of the hog business temporarily.

CHAIRMAN GWATKIN.—Any other questions? We are keeping well within our time, so we have plenty of time for discussion.

DR. JOHNSON.—What did you do, Dr. Doyle, about disinfecting the premises?

DR. DOYLE.—We used the same procedure that we ordinarily use, applying either pine oil or liquor cresolis.

We think the dangerous factor in the spread of this disease is the hog that has been exposed to the disease and has recovered. We have circumstantial evidence to indicate that some recovered animals are carriers.

When we break the contact between the healthy hog and the one with dysentry, we have gone a long way toward preventing further spread of the disease. Then, if we can get rid of all carriers, we can control the disease effectively.

DR. CHARLES H. BECKMAN (St. Louis, Mo.).—Do you have an increase in the degree of virulence and the number of new cases in wet, damp, soggy weather?

DR. DOYLE.—We have not been able to correlate the fluctuation in incidence with weather conditions; however, as in hog cholera, as soon as the traffic in hogs becomes great in any one area we are very likely to have an increase in the incidence of dysentery.

Another factor which probably influences the incidence of seriousness of the disease is the age factor. It is more destructive in younger hogs than in older ones. We have an increased prevalence of it during the time of year in which we have a large number of young hogs.

The Effect of Environment on the Survival of the Free-Living Stages of Trichostrongylus Colubriformis and Other Nematode Parasites of Range Sheep in Southeastern Montana

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PARASITISM of range sheep has been considered of minor significance, but in recent years increasing reports have been received in midsummer from eastern Montana and western North and South Dakota of diarrheic lambs and, not infrequently, of unthrifty lambs being the result of intestinal parasitism. At the request of the sheepmen, a number of surveys and investigations have been conducted during the last five years in a part of this area lying in southeastern Montana.

The parasites most frequently observed were *Ostertagia circumcincta, Trichostrongylus colubriformis, Nematodirus spathiger, Moniezia expansa,* and not infrequently *Haemonchus contortus* in small numbers. Infections with Moniezia are common in eastern Montana lambs, but from observations over a period of years, tapeworms cannot definitely be associated with widespread diarrhea in range lambs. Little specific evidence is available as to the pathogenicity of Nematodirus. Heavy infestations of this parasite are infrequent in lambs scouring in the middle of the summer, although, later in the season heavy infections of Nematodirus are observed in some diarrheic lambs. *O. circumcincta,* which occurs more frequently than any other nematode in range lambs, has not been found in sufficient numbers to be the cause of this trouble.

The most typical, though not constant, symptom of trichostrongylosis in sheep is diarrhea. Owing to the difficulty of proper interpretation of low egg counts and of evaluation of low worm counts in most of the cases of diarrheic lambs observed in our investigations, it was evident that, in order to ascertain the significance of Trichostrongylus in range lambs, controlled experiments with worms of this genus were necessary.

For our immediate information, a series of experiments was planned for the biologic testing of the range under controlled conditions in southeastern Montana, during the period the lambs are with the ewes. The experiments reported were carried on in 1945 and 1946 at Albion, Mont., in the southeastern part of the state.

Investigations relative to the effect of climatic and environmental conditions on the survival of eggs and the free-living stages of sheep parasites, under natural conditions, by the use of worm-free sheep are comparatively few. Detailed experiments have been carried out in eastern United States, principally by the Zoölogical Division of the U. S. Bureau of Animal Industry. The results of these experiments are of much practical value, but they fail to give definite information on the more important species affecting sheep in the Great Plains area, where the environmental conditions differ from those of eastern United States. Although nearly all of the commonly known species of nematode parasites of sheep were involved, particular emphasis has been placed on *H. contortus* because of its importance in that section of the country. In the eastern part of Montana, Haemonchus is of minor importance as a causative factor of parasitism in sheep, whereas in western South Dakota, which has a higher rainfall, it is of greater significance.

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ENVIRONMENTAL FACTORS

In general, the topography in eastern Montana is characterized as a high semi-arid plain, dissected by rivers and their tributaries, most of which have a continuous flow during the spring and early summer, but later in the season they are likely to disappear in the stream gravels or shrink into a number of water holes. The land is largely residual soils washed from shale. The heavy textured soils, known locally as gumbo, tend to impede internal drainage. The sheep are mostly watered from reservoirs which are filled by surface drainage. In general, these reservoirs are devoid of luxuriant growth of vegetation and swampy areas along the shoreline. The physiographic features of the area are shown in figure 1.

The native vegetation is a short grass association with broken sod. The dominant grasses are western wheat grass, blue grama, buffalo grass, "niggerwool", and June grass. Groves of cottonwood and box elder trees border the larger streams. Sagebrush and cactus are common. The land has a carrying capacity of 1 ewe and lamb to 6 to 8 acres of range.

The average annual precipitation is approximately 13 in. occurring mostly in May and June. The precipitation differs considerably from year to year, as does the distribution throughout the area during the growing season. Seasonal and daily temperatures are subject to wide extremes.

These factors influence the development and survival of the preparasitic stages of sheep nematodes, and for this reason the weather conditions that occurred during the experiments are recorded. The meteorologic data were obtained from the U. S. Weather Bureau Station at Albion, Mont. The data in table 1 show the means of the temperature and the rainfall during the time the plots were being infested and for the interval of ten days allotted for the development of the free-living stages. The weather conditions that prevailed throughout the experiments are shown in table 2. No records

TABLE 1—Summary of Weather Data Collected at Albion, Mont., During the Infestation of the Plots and Interval of Time Allotted for Development of the Larvae

	Temperatures (F.)					Precipitation (inches)		
	Mean	Max. mean	Extreme high	Min. mean	Extreme low	Total	Days	Greatest in 24 hr.
1946								
Plot 1								
May 10-May 24	51.4	62.0	76	40.8	25	3.36	5	2.23
1945								
Plot 2								
June 11-June 25	58.3	71.6	90	44.9	35	0.98	7	0.47
Plot 3								
July 15-July 29	76.4	92.0	101	61.8	59	0.89	4	0.59
Plot 4								
Aug. 2-Aug. 18	74.1	88.5	101	59.8	47	0.93	5	0.62

TABLE 2—Summary of Weather Data Collected at Albion, Mont., During the Time of the Experiments

	Temperatures (F.)			Precipitation (inches)		
	Mean	Max. mean	Min. mean	Total	Days	Greatest in 24 hr.
1945						
June	55.3	66.3	44.3	1.84	14	0.47
July	73.1	87.7	58.5	1.58	9	0.59
August	71.6	88.4	54.8	0.97	6	0.62
September	55.4	68.9	42.0	2.55	5	1.08
October	47.4	63.3	31.5	0.23	2	0.19
November	27.8	40.1	15.6	0.35	1	0.35
December	19.4	29.3	9.5	0.22	3	0.17
1946						
January	26.2	38.8	13.6	0.08	1	0.06
February	27.7	42.0	13.5	Trace	0	Trace
March	39.0	52.6	25.5	1.80	2	1.60
April	50.0	66.0	34.0	0.68	3	0.39
May	50.2	61.1	39.3	5.18	8	2.23
June	64.7	76.7	52.8	4.87	6	2.20
July	71.1	86.8	55.4	Trace	0	Trace
August	65.8	82.1	49.6	1.23	5	0.65

were available of the relative humidity at Albion, although it does not differ materially from other localities in the eastern division of the state. The seasonal evaporation rate at Fort Peck, in northeastern Montana, from May to October during 1945 and 1946 was well above 30 in., which is indicative of a low relative humidity.

MATERIALS AND METHODS

The plan of the experiments is similar to that used by Shorb[1] in testing the survival periods of infective larvae under natural conditions. Groups of worm-free lambs were placed successively on previously infested plots at intervals throughout the summer months, and observations were made on resulting infections by means of fecal egg counts.

The experimental lambs which were raised worm-free were selected from ewes with twins. At parturition, they were placed in cleaned individual pens for two days to acquaint the ewe with her lambs and to insure an ample milk supply. Then the ewe and lambs were moved to a concrete-floored shed, bedded with straw. Since the time required for the development of the free-living stages of sheep nematodes is brief, under optimum conditions, the ewes and lambs were removed to a clean floored corral twice a week, while the floor of the permanent shed and the feed troughs were cleaned, washed, and left to dry. The floor was then bedded with clean straw before the animals were returned. The ewes and lambs were fed alfalfa hay supplemented with whole oats which were placed in elevated troughs. Phenothiazine and salt (1:9) and water were available to them at all times. This method of raising lambs free of worm infection has proved satisfactory. No nematode eggs were observed in the lambs raised except Strongyloides and occasionally a few Nematodirus. The animals which became infected with Nematodirus were not used for experimental purposes. The advantage of raising worm-free lambs, as described, over the method of separating the lambs from the ewes at parturition and raising them on a bottle is that a large number of worm-free lambs may be raised with minimum labor and equipment.

Fecal examinations were made of the lambs at monthly intervals beginning when they were 4 weeks of age. Examinations were also made immediately before and after exposure of the lambs on infested areas. The acquired infections in exposed lambs were determined by egg differentiation and checked by fecal cultures twenty-one days after removal from infested areas. One of the 2 lambs used for each exposure was destroyed to determine the number of worms it harbored and to confirm the species. Lambs were considered negative when consecutive fecal examinations failed to show the presence of any nematode eggs, except Strongyloides.

The method used for examination of fecal samples was a modification of the Lane centrifugal flotation method as described by Stoll.[2] The principal difference is in the use of a sucrose solution, 100 Gm. of sucrose to 100 cc.

TABLE 3—Summary of Fecal Egg Counts of Worm-free Lambs Three Weeks After Exposure on Infested Plots

Experimentally infested plots	Date plot infested	Sub-division	Lamb	Interval of exposure	Fecal egg count/gram
				1946	
Plot 1	May 13-May 16	A	R-50	May 24-June 12	190
			R-127		40
		B	R-123	June 25-July 26	75
			R-134		145
		C	R-22	July 24-Aug. 8	0
			R-125		0
				1945	
Plot 2	June 11-June 14	A	677	June 25-July 6	410
			676		120
		B	681	July 29-Aug. 13	25
			683		10*
		C	R-4	Aug. 18-Sept. 5	10
			R-56		10
				1945	
Plot 3	July 15-July 19	A	684	July 29-Aug. 13	0
			686		0
		B	R-5	Aug. 18-Sept. 5	0
			R-27		8*
				1945	
Plot 4	Aug. 2-Aug. 8	A	R-52	Aug. 18-Sept. 5	0
			R-26		0
		B	R-53	Sept. 20-Oct. 18	10*
			R-59		5*

*Nematodirus eggs only.

of water, instead of salt for the flotation medium. Ten-gram fecal samples, collected rectally, were used throughout, except when fecal egg counts were desired on larger numbers of sheep. In obtaining the fecal egg count of a band of sheep, individual pellets were obtained from 200 or more animals and pooled.

The experiments were carried out on range which is typical of that used by sheep operators in this section of the state. The land used had not been grazed by sheep or other livestock for several years, and samples of vegetation and soil examined by the Baermann technique were negative for parasitic larvae. The area was divided into a series of fenced plots 40 by 120 ft. The plots were to be infested successively throughout the spring and summer, when lambs were with the ewes. Unavoidably, the first plot was not infested as was planned near lambing time, in the spring of 1945, and the work had to be completed in tne spring of 1946, to cover the original plan of experiments.

Ten yearling sheep were used for infestation of the plots. They were predominantly infected with *T. colubriformis* but carried insignificant numbers of Ostertagia and Nematodirus, and very few Haemonchus, as evidenced by egg differentiation and fecal cultures. They were further parasitized by daily doses of infective *T. Colubriformis* larvae. The fecal counts for the lot averaged 1,400 eggs per gram. Excluding one low count of 500, they varied from 1,000 to 2,500. The plots were grazed for three days and vacated for ten days to allow for the hatching of the eggs and development of the larvae. Then the plots were subdivided by fence into three subdivisions for subsequent test exposures with worm-free lambs. At the time worm-free lambs were first placed on a plot, it was tested for infective larvae by random sampling of vegetation and soil.

The first pair of lambs was placed on the plot ten days after the removal of the yearlings. After the first exposure, the subdivisions of the plot were tested at monthly intervals. The lambs were held on each subdivision of the plots for a maximum of two weeks, but for less time in some cases due to the lack of forage. Two worm-free lambs were used in each of the exposures in the experiments. At the end of the exposure period, the lambs were transported to concrete-floored pens where they remained for sufficient time to allow for maturity of any larvae that may have been ingested.

At the same time the lambs were exposed on each of the respective plots, 2 worm-free lambs were placed with a range band in the immediate vicinity for two weeks. This was to obtain information relative to the pick up of infective larvae on the range at corresponding periods. The lambs exposed were of an age comparable to the range lambs.

In addition, 2 lambs were placed with a range band in early December to obtain possible information on the relative pick up of infective larvae on the range during late fall and early winter.

Fig. 1 — Type of sheep range in eastern Montana.

RESULTS OF EXPOSURE ON EXPERIMENTALLY INFESTED PLOTS

Plot 1.—Plot 1 was infested by the yearlings from May 13 to May 16, 1946. The 2 lambs, which were grazed on subdivision A of plot 1 from May 24 to June 10, developed light infections as indicated by fecal counts of 190 and 40 eggs per gram, respectively. Those exposed from June 25 to July 24, 1946, in the second subdivision of the same plot, showed similar infections. Exposure on the third subdivision of this plot from

July 24 to Aug. 8, 1946, resulted in negative fecal counts for both lambs.

Plot 2.—Lambs were placed on subdivision A of plot 2 (which was infested by the yearlings from June 11 to June 14, 1945) from June 25 to July 6, 1945, and subsequent fecal examinations showed 410 and 120 eggs per gram, respectively. One of the lambs (677) showing a fecal count of 410 eggs was destroyed. At autopsy, 8

TABLE 4—Summary of Fecal Egg Counts of Worm-free Lambs Three Weeks After Exposure with a Band on the Range

Lamb	Interval of exposure on the range	Fecal egg counts/gram
	1945	
R-6	June 25-July 6	610
R-7		220
R-9	July 29-Aug. 13	5*
R-58		50
R-24	Aug. 18-Sept. 5	0
R-57		2*
R-11	Nov. 30-Dec. 23	60*
R-12		94*
	1946	
M-449	May 24-June 6	30
M-450		170
M-431	June 25-July 26	920
M-432		250
M-447	July 24-Aug. 6	0
M-448		0

*Nematodirus only.

Haemonchus, 32 Ostertagia, and 2,000 Trichostrongylus were recovered. The lambs exposed on the two remaining subdivisions from July 29 to Aug. 13, and from Aug. 18 to Sept. 3, 1945, developed fecal counts of 15 and 30 eggs per gram, and 25 and 5 eggs per gram. As evidenced by the fecal counts, these lambs had acquired very light infections. Lamb R-4 exposed from August 18 to September 3, was killed, and it showed only 68 Trichostrongylus and 70 Nematodirus.

Plot 3.—Exposure of lambs on plot 3 (which was infested by the yearlings from July 15 to July 19, 1945) was made from July 29 to August 13, and from August 18 to September 3. All of these lambs showed negative fecal egg counts following exposure, except for a few Nematodirus eggs. At autopsy, lamb 684, of the first exposure on plot 3, showed 13 Trichostrongylus, 2 Nematodirus, and no Haemonchus. Lamb R-27, of the second exposure, showed a negative fecal count, except for Nematodirus eggs. At autopsy, only 6 Trichostrongylus and 5 Nematodirus were recovered.

Plot 4.—On plot 4 (which was infested by the yearlings from Aug. 2 to Aug. 8, 1945), the lambs were exposed from August 18 to September 3, and September 20 to October 20. The results obtained on plot 4 were similar to those of plot 3. Lamb R-52, of the first exposure, showed 4 Trichostrongylus and 2 Nematodirus, and lamb R-59, of the second exposure, showed only 10 Trichostrongylus at autopsy. A synopsis of the fecal examinations of all of the lambs exposed on the experimental plots is shown in table 3.

RESULTS OF EXPOSURES WITH RANGE BAND

The supplemental information obtained on exposing parasite-free lambs with a range band closely coincided with the results obtained from the plot experiments. The band of sheep with which the lambs were exposed showed a higher incidence of Haemonchus than is generally found in Montana range sheep. A fecal count of some 400 ewes and lambs showed 1,300 eggs per gram. They were differentiated as 61 per cent Haemonchus, 3 per cent Ostertagia, 27 per cent Trichostrongylus, and 9 per cent Nematodirus. In evaluating the degree of infection by fecal counts, it must be remembered that *H. contortus* is notably a high egg-laying species.

One of the lambs (R-6) which was placed on the range from June 25 to July 6, 1945, showed a subsequent fecal egg count of 610 eggs per gram. At autopsy, 80 Haemonchus, 60 Trichostrongylus, and 30 immature Nematodirus were recovered. The lambs exposed from July 29 to August 13 and those exposed from Aug. 18 to Sept. 5, 1945, acquired light infections. Lamb R-57, from the latter exposure, showed only a few Nematodirus eggs on fecal examination, and at autopsy only 36 Nematodirus were recovered. The fecal egg counts of lambs placed with the range band in the late spring and summer of 1946 closely correlated with the results obtained in 1945. The heaviest infections were acquired in June

of both years. The results of the fecal egg counts of all the lambs on range exposure are tabulated in table 4.

Exposure of worm-free lambs in the late fall on range which had not been used since lambing resulted in negative fecal counts, except for high numbers of Nematodirus eggs. Unavoidably, the lambs were held with the band longer than two weeks, but they failed to develop any infection other than Nematodirus. During the twenty-three days of exposure, the maximum temperature varied from 4F. to 53F., and the minimum from –20F. to 28F. The mean temperature for the period was 16.9F., and the precipitation totalled 0.17 in., which occurred on the last two days of exposure.

SURVIVAL OF EGGS AND LARVAE ON RANGE DURING LATE FALL, WINTER, AND SPRING

Dikmans[3] and others [4, 5] have shown that larval forms of gastrointestinal parasites of sheep may survive over winter and retain their infectivity. A field experiment was conducted to determine the effect of winter conditions on the longevity and availability of the free-living stages of range sheep nematodes in southeastern Montana.

A plot of range was selected that was apparently free of any infective larvae, as indicated by the Baermann technique applied to random samples of the sod and grass. Fourteen rams from a lot of 26 that showed an average fecal count of 2,400 eggs per gram were selected for infestation of a fenced plot 20 by 60 ft. An autopsy of 1 of the rams showed the following species and numbers of worms: 4,000 Haemonchus, 250 Ostertagia, 28,500 Trichostrongylus, and 2,400 Nematodirus. The animals were left on the plot for only one day, Nov. 30, 1944, in an attempt to simulate the fecal contamination found on the range. The weather conditions that prevailed in the general area from December, 1944, to June, 1945, are shown in table 5. The weather data were compiled by averaging observations made at Broadus and Ekalaka, Mont., since the weather station at Albion, Mont., was not established until June, 1945. It is realized that weather conditions are variable, and that these meteorologic data give only an approximate picture of the weather at the experimental plot.

On June 11, 1945, seven months after infestation of the plot, 2 worm-free lambs (A-11 and A-5) were grazed on the plot for two weeks and then removed. Fecal examinations made three weeks after the removal of the lambs from the plot showed both animals to be infected. Lamb A-11 had a fecal count of 45 eggs per gram, 20 of which were Nematodirus. The other lamb (A-5) showed 520 eggs per gram, 40 of which were Nematodirus. At autopsy, 30 Haemonchus, 80 Trichostrongylus, and 120 Nematodirus were recovered from lamb A-5.

Fig. 2—Experimental area. Parasitized yearlings grazing one of the plots.

One year and seven months after the plot had been infested, 2 worm-free yearlings were put on the plot and removed after grazing it for one week. Three weeks following exposure, both yearlings showed very light infections. At autopsy, yearling R-129 showed 10 Ostertagia, 120 Nematodirus, and no Trichostrongylus.

SHEEP RESERVOIRS AS A POSSIBLE SOURCE OF NEMATODE INFECTIONS

In eastern Montana, stock reservoirs are so located on the range that they are replenished with water from surface drainage of many acres of range. The run-off resulting from rains or cloudbursts is tremendous. Therefore, it seems logical to assume that untold numbers of eggs and larvae are washed into the reservoirs. This matter has been of some concern in sheep management. The available information, which is based largely on laboratory studies where eggs or larvae have been immersed in varying depths of water, indicates that the eggs or larvae of parasitic nematodes would survive for only a short time in such bodies of water.

In general, the shorelines are of minor significance as sources of infection. Reservoir shorelines are highly inconstant due to the rapid rate of evaporation. As a rule, they support a minimum growth of unpalatable forage and are devoid of swampy areas.

During the past two years, a number of samples of reservoir water has been obtained during the growing season, under varied climatic conditions from the shallow water at the shoreline. They have been examined for the presence of eggs and parasitic larvae by the Baermann technique with negative results. Parasite-free sheep drenched with some of the samples of reservoir water remained worm-free.

Late in August, 1945, a reservoir was selected which afforded an opportunity to test the validity of the supposition that water is a possible source of nematode infections in the field. This reservoir had been the only source of water for a band of ewes and lambs since they were placed on the range in June. A composite fecal examination of some 200 animals out of the 400 ewes and their lambs in this band showed 1,000 eggs per gram, differentiated as 70 per cent Haemonchus, 26 per cent Trichostrongylus, 2 per cent Ostertagia, and 2 per cent Nematodirus.

The south shore of the reservoir was selected because a considerable area bordering the reservoir was devoid of any vegetation. A corral, extending into the water, was built large enough to accomodate 2 lambs. A suitable feed rack was provided for hay. The water of the reservoir was at a low level. However, a week after the lambs were placed in the corral, 1½ in. of rain fell in less than twenty-four hours, replenishing the reservoir. The lambs remained in the corral for two weeks and then were moved to the laboratory. Subsequent fecal examinations three weeks later were negative, except for small numbers of Nematodirus eggs. One of the lambs (R-25) was destroyed, and it harbored 7 Trichostrongylus and 275 Nematodirus. Since the infective larvae of Nematodirus may survive in water from five[6] to eleven months,[7] this may account for the increased numbers of Nematodirus in the lambs as compared with Trichostrongylus.

This result indicates that the greater percentage of the nematode eggs or the

TABLE 5—Summary of Weather Data Compiled for the Period of the Over-Winter Survival Experiment

Month	Temperatures (F.)					Precip. (inches)	
	Mean	Maximum mean	Extreme high	Minimum mean	Extreme low	Total	Days
1944							
December	22.1	34.8	49	9.4	—13	0.42	6
1945							
January	21.8	33.3	47	10.3	—26	0.70	9
February	22.6	34.1	48	11.1	—17	0.37	8
March	34.8	47.5	71	22.1	—27	1.25	9
April	40.3	53.3	75	27.3	7	0.73	5
May	50.6	63.6	79	37.6	15	1.57	9
June	56.4	67.4	93	45.4	33	1.85	15

larvae washed into reservoirs by surface drainage do not survive for any length of time. As a result, it is believed that the danger is not so much from the reservoirs themselves as from the resulting concentration of fecal contamination on the surrounding grazing area, which the sheep graze as they move from water to feed.

DISCUSSION

A factor that must be considered in the interpretation of the results of the plot experiments is that the yearlings used for infestation were not as heavily parasitized as was desired, although the fecal egg counts showed that they carried heavier infections than are generally observed in range sheep. Some of the irregularities in the execution of the experiment, where lambs were held on plots or the range for longer than two weeks, were due to conditions indirectly resulting from conducting controlled field experiments some 500 miles from the laboratory. Lambs which were left unavoidably on range or plots for longer than two weeks did not build up relatively heavier infections.

The results of the experiments conducted on the plots and on the range show that infective larvae of Haemonchus and Trichostrongylus rapidly disappear from the range after the spring rains. Haemonchus larvae were found to be more susceptible to environmental conditions. Trichostrongylus larvae were more resistant than Haemonchus, yet their numbers diminished rapidly on the range after mid-July. The infective stages of Ostertagia and Nematodirus were the most resistant to environmental changes. Monnig[8] has shown that a few weeks of summer dryness are sufficient to kill the infective larvae of *T. colubriformis* on heavy soils, because the larvae are not able to migrate to sufficient depth for adequate protection.

The results obtained by contaminating a clean range plot in early winter and letting it remain idle until early summer showed that a small number of Haemonchus, Ostertagia, Trichostrongylus, and Nematodirus survived winter conditions and developed into infective larvae, as evidenced by infections in worm-free lambs grazed on the area. It is realized that the results are not quantitative, although it is believed that most of the eggs or preparasitic stages did not survive over winter, as shown by the the resulting light infections in the lambs grazed on the plot in June. These observations are in accordance with the work of Monnig[8] on Trichostrongylus, Furman[9] on *O. circumcincta*, and Dinaburg[10] who showed that these genera will resist low temperatures for considerable periods. Unpublished data from this laboratory on *N. spathiger* show that eggs of Nematodirus contained in feces will develop complete embryos in winter when placed on the ground with no protection. After four months exposure, all of the eggs hatched to infective larvae when placed under optimum conditions indoors. In this experiment, the eggs were exposed from Dec. 20, 1943 to April 21, 1944. The maximum temperature varied from 10F. to 67F., and the minimum temperature from –14F. to 40F. The mean temperature was 25.9F. and the precipitation was 2.18 in. for the period.

The results in regard to the over-winter survival of *H. contortus* are somewhat at variance with Dinaburg's work[11] on Haemonchus, who has shown that outdoor temperatures were the limiting factor in the development and survival of the eggs and larvae of *H. contortus* at mean maximum temperatures below 65F. In this experiment, a small number of *H. contortus* eggs or infective larvae survived winter conditions more severe than those recorded by Dinaburg, and seven months after infestation of the plot the larvae were infective.

The lambs which were exposed with a band on winter range for three weeks in December, 1945, developed moderate infections of Nematodirus only, as evidenced by repeated fecal counts. This experiment indicates that the eggs deposited on range do not develop to the infective stage when low temperatures prevail, except Nematodirus.

SUMMARY AND CONCLUSIONS

The development of the eggs and survival of the infective larvae of the nematodes parasitic in the intestinal tract of

range sheep, particularly the Trichostrongylus species, under the influence of natural weather conditions of southeastern Montana, were studied by exposing worm-free lambs on preinfested plots and range. The experiments were carried on from June, 1945 to August, 1946, at Albion, Mont.

From the foregoing data, it may be concluded that infections of range lambs with gastrointestinal nematodes are acquired principally during May and June, when the heaviest rainfall generally occurs on the range. In these experiments, the range became practically free of the infective larvae of Haemonchus and Trichostrongylus after mid-July. During the latter part of the summer, when less than 1 in. of rain fell in a two-month period, the majority of the eggs and preparasitic stages of the nematodes common to range sheep were killed within ten days' exposure on the range.

Under range conditions in late fall or early winter, there was no development to infectivity of the free-living stages of Haemonchus, Ostertagia, or Trichostrongylus, but the eggs of Nematodirus developed into infective larvae, as evidenced by pure infections in worm-free lambs.

A small number of Haemonchus, Ostertagia, Trichostrongylus, and Nematodirus survived over winter, either in the egg stage or as infective larvae, as evidenced by infections in lambs seven months following deposition of the eggs on the range.

Evidence is presented that reservoirs are of no importance as a direct source of nematode infections in range sheep.

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The Development of the Dog Ovary from Birth to 6 Months of Age

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Review of the Literature

Chronologically, the recognition of, and report on, the ovaries as specific entities was first made by Baer[2] in 1827. Waldeyer's[15] research on the canine ovary with the observation of Pflüger's cords actually began the investigation in this species.

It has been reported in the works of numerous authors, Allen,[1] Barton,[3] Duke,[5] Evans and Cole,[6] and Kingsbury,[13] that the primordial sex cells of the ovary arise from the secondary sex cords (cords of Pflüger).

The derivation of the earlier cords, the primary sex cords or medullary cords, has been a disputed point. The classical belief, upheld by Jonckheere,[12] was that medullary cords originated from the germinal epithelium, specifically as the first organogenetic bud. Gruenwald[9] opposed this view as he contended that the primary sex cords arose *in situ* from differentiated mesenchyma during a period in which the epithelium had lost its entity and was functioning with the mesenchymal blastema as a single unit in the production of the medullary cords.

Hartman[10] observed polyovular follicles in the ovaries of the dog. Stafford *et al.*[14] described the thecal gland in the dog ovary and recognized it at the time of formation of the vesicle and in the follicle. Evans and Cole[6] reported the first polar body of the dog ovum to be released following ovulation and after the ovum was in the Fallopian tube.

In the dog, Evans and Swezy[7] showed ovogenesis to occur throughout the whole period of sexual life and in a rhythmic sequence which they termed the "follicular cycle."

Objectives

The tissues were studied with the view of determining:

1) Morphologic changes in the germinal epithelium and its component cells.

2) Developmental changes in the formation of the tunica albuginea.

3) Regressions in the layer of parenchymatous cortical cells.

4) Formation and locations of follicles.

5) Variation in the structure of the corticomedullary junction.

Method of Procedure

The ovaries of 37 dogs were used in this study. Upon complete exsanguination through section of the carotid artery, the individual visceral organs were removed from the body cavities, along with a complete set of endocrine glands. The tissues were stripped of extraneous fat and weighed. Following fixation with either Bouin's or 10 per cent formalin and embedding in paraffin, the tissues were sectioned at 10 μ. Heidenhain's iron hematoxylin, Delafield's hematoxylin, Harris' hematoxylin, and Mallory's triple stain were used with the latter stain giving the most satisfactory results in the writer's hands.

Results

The observations reported on all sections were made by describing the tissue of the ovary from the periphery inwardly.

Two Days Postnatal.—The germinal epithelium was composed of relatively large, cube-shaped cells containing well-defined ovoid or elongated nuclei. Below this an irregular layer of connective tissue, for the most part only one cell thick, was broken through by deeper cell groups still maintaining epithelial contact. Among these deeper cell groups lay what appeared to be the precursors of the follicle cells and ova. The follicle cells were composed of a voluminous and granular cytoplasm containing large nuclei. Within some nuclei were evidences of mitotic activity.

Beneath these cell groups lay a second stratum of connective tissue. Next appeared what was interpreted to be remnants of an earlier cortical ingrowth (primary sex cords) of smaller cells with darker staining nuclei.

Six Days Postnatal.—A thick, single-celled connective tissue basement membrane was well defined. At some points, radiating connective tissue strands arising deeply in

From the Department of Veterinary Anatomy, Division of Veterinary Medicine, Iowa State College, Ames. Taken from a thesis submitted to the Graduate College in partial fulfillment of requirements for degree of Master of Science (1946).

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the ovary had connected with the basement membrane. Between these strands the cell nests and cordlike formations of cells were easily identified.

The cord formations were composed of cells differentiated into both larger spherical nucleated cells and smaller ovoid cells containing flattened nuclei. In the medulla,

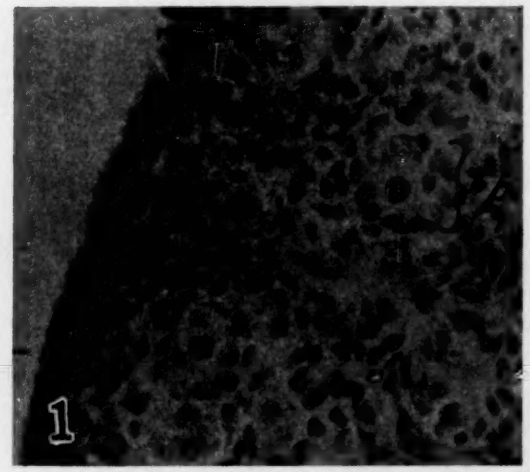

Fig. 1—Four days postnatal. (a) Cuboidal cells of germinal epithelium; (b) precursor of oöcyte with surrounding primordial follicle cells.

fine parenchymatous tissue cords were composed of primarily the smaller ovoid types.

Seventeen Days Postnatal.—A heavy tunica albuginea several cells thick had developed below the squamous cell layer of germinal epithelium. At some points the tunic was perforated by groups of ingrowing germ cells extending from the epithelium to the cortex. The nuclei of the germ cells showed evidence of mitotic division.

Eleven Weeks Postnatal.—Cuboidal cells had reappeared in the germinal epithelium (fig. 2, b). Below it the tunica albuginea remained six or more cells deep. Connective tissue strands connected it with the deeper ovarian stroma and separated the cell groups in the cortex which now was a very narrow zone.

The cell groups (fig. 2, a) formed complete cords of primordial ova with individual complements of granulosa cells extending to the superficial surfaces of the medullary zone.

Fifteen Weeks Postnatal.—The subepithelial zone of parenchymal cell activity was reduced to a depth of three to five cells. The connective tissue in this portion of the cortex had increased greatly. For the first time the primordial germ cells were surrounded by a stratified granulosa. Some follicles already showed evidences of degeneration by the appearance of their nuclear material. The karyoplasm was not evenly distributed, giving the nucleus the appearance of being vacuolated.

Twenty Weeks Postnatal.—The germinal epithelium appeared only as a single layer of squamous shaped cells.

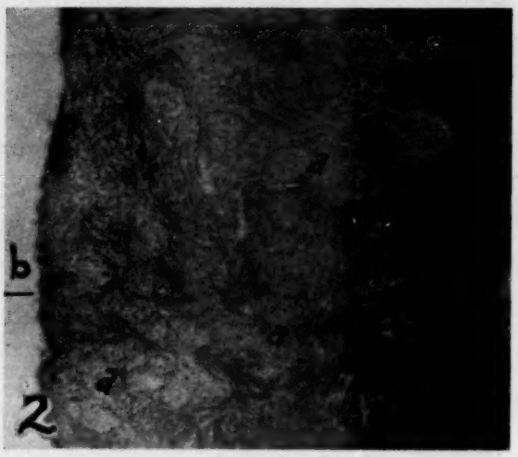

Fig. 2—Eleven weeks postnatal. (a) Cell cords of primordial ova; (b) cuboidal cells in germinal epithelium.

The secondary sex cords (fig. 3, a) were principally composed of primordial ova, closely packed to form dense cords. Their nuclei were large with chromatin material dispersed along the nuclear membrane.

The primordial ova whose granulosal cells had begun active proliferation were located toward the deep end of the cords. Many of these ova showed evidence of disintegration with retraction of their nuclear membranes leaving vacuoles in the cell substances. Cytoplasmic granules were not evenly dispersed throughout the cells but had left a clear zone near the cell membrane.

Six Months Postnatal.—The germinal epithelium was composed again of well-defined cuboidal cells. At intervals, areas of small masses of active cells protruded

from the deep surface of the epithelium and extended into spaces of the albuginea. Below the tunic lay small nests of primordial ova which in some areas formed cords with their long axes perpendicular to the ovarian surface. The ovocytes contained in these cords were surrounded by a layer of follicle epithelium cells, one and two cells deep.

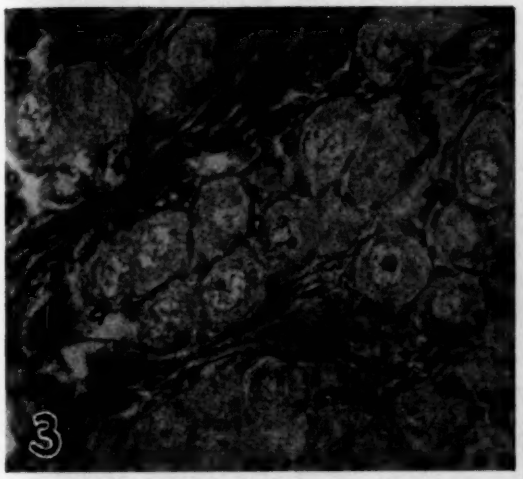

Fig. 3—Twenty weeks postnatal. (a) Secondary sex cords; (b) cellular arrangement suggesting future lumina development.

Next to the cords were the first formations of antrums seen within follicles. these appeared due to the separation of the granulosa cells leaving small, clear areas (fig. 4, a). Organization of the theca as an entity from differentiated medullary stromal cells was also seen at this period.

DISCUSSION

The literature reviewed made no reference to the first occurrence of the granulosal stratification about the newly formed ova. This is reported here to occur at the fifteenth week postnatal age. Its importance cannot be overestimated for it serves as a well-defined landmark in the growth study of the follicle. A second developmental stage not previously reported in this species is that of the antrum formation in the immature follicle. This had been reported in the pig and calf by Casida.[4]

A study of ova of the next age group beyond the scope of this thesis showed the coalescing of the small antrums into the true follicular cavity.

Although the medulla was involved in little activity during this period of study, its peripheral area was quite active. Additions to it in the form of secondary sex cords from the epithelium soon dispersed the well-defined cortico-medullary junction. Also effective in destroying the line as a definite junction was the number of connective tissue strands which emanated from it about the ninth week.

The germinal epithelium presented a number of changes in cell types with the different ages examined. The young post-

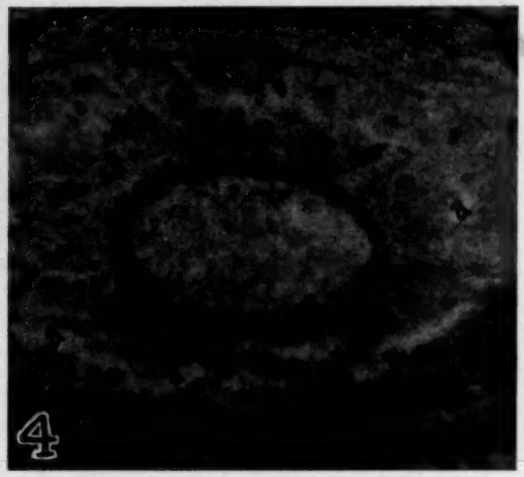

Fig. 4—Six months postnatal. (a) Beginning antrum formations; (b) developing theca from adjacent stroma cells.

natal ovary had cuboidal cells in the epithelium. In ten days these were being replaced and had become a squamous type by the thirteenth day, remaining so until the sixty-third day. For a two-week period the cell type again was predominantly cuboidal to regress by the fifteenth week to the squamous cells.

Barton[3] has correlated the germinal epithelium cell type with ovarian activity, and in this study similar conclusions could be drawn, for the greatest ingrowth of cords was made in the presence of the cuboidal cells.

The first appearance of Pflüger's tubes was noted at 15 days postnatal growth, coincident with the first follicle formation.

Evans and Swezy[7] have correlated this growth with portions of the estrual cycle, and one might speculate that this very immature ovary is already beginning abortive estrual cycles.

A subepithelial connective tissue layer, anlage of the tunica albuginea, was present in the earliest tissues studied. An interesting speculation can be made on the source of material for its growth into the true tunic. This development appeared as the connective tissue in the medulla, which, through its radiating strands of cells, contacted the tunic and actually supplied tissue cells to it through this newly formed function.

From the observations made and in concurrence with Everett,[8] the work of Harz[11] could not be supported, namely, that the tunica albuginea functioned as the regulating tissue for the formation of ova.

SUMMARY AND CONCLUSIONS

The time of appearance of the following specific changes in the dog ovary have been recorded from a study beginning with the two-day postnatal stage and extending into the sixth month.

1) Development of the follicle.

a) Primordial ovocytes surrounded by follicle epithelial cells were seen at 4 days of age.

b) True primary follicle formations with granulosa cell development from the follicle cells occurred at about 15 days.

c) Stratification of the granulosa became apparent at 15 weeks.

d) Antrum formation was not observed until 6 months of age.

2) Germinal epithelial activity was not a continuous process but appeared to occur cyclically.

3) The development of the tunica albuginea reached its greatest peak in early life when the tunic received tissue contributions from the medulla.

4) Cortical activity was greatest at about 13 days of age and seemed to be inversely correlated with medullary activity.

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Species Susceptibility to the Viruses of Carré and Feline Enteritis

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IN CONSIDERING the virus of Carré (canine distemper), it appears that it is not naturally species specific as is the virus of hog cholera, nor does it have a wide range of susceptible hosts as equine encephalomyelitis virus. The equine virus has been proved to attack guinea pigs, white mice, calves, man, horses, and a wide range of birds either naturally or by experimental inoculation. The known range of susceptibility to Carré's virus is, as will be seen, much more restricted.

VIRUS OF CARRE

Carré's virus does not fit into the loose classification of viruses of Topley and Wilson. They divide viruses into four groups:

1) Characterized by lesions of the skin.

2) Characterized by lesions of the central nervous system.

3) Characterized by catarrhal or generalized lesions.

4) Characterized by tumor formation.

In the case of the virus of Carré, the lesions in the dog may be predominantly those of the central nervous system. The same virus inoculated into ferrets may produce generalized lesions, including those of the skin. Seemingly, the type of lesions produced by this virus depends somewhat on the species infected.

Armstrong and Anthony[1] described an outbreak of canine distemper in the National Zoölogical Park in Washington, D. C. The disease occurred in a unit housing both Canidae and Felidae in adjoining buildings. Only the Canidae were affected.

These same authors state that Hamerton observed distemper in a binturong (*Arctitis binturong*) in an outbreak in the Canidae in the London Zoo. Hamerton's article [Proceedings of the Zoölogical Society of London, *2*, (1937) :443] does not mention the binturong as one of the affected species in the distemper outbreak reported by him.

At the New York Zoölogical Park we have, from time to time, had outbreaks of distemper and have only seen it once in animals other than Canidae and Mustelidae. In 1943, several binturongs, of the family Viverridae, sickened and died with symptoms like those of canine distemper. The symptoms consisted of purulent conjunctivitis and rhinitis, diarrhea, high temperatures, and inappetance. The gross lesions were gastroenteritis, pneumonia, tracheitis, and rhinitis. Intranuclear and cytoplasmic inclusions were numerous. Figure 1 shows intranuclear inclusions in the cells of the kidney tubules. No attempts were made to transmit the virus to other animals.

TABLE I—Canine Distemper

Order Carnivora—flesh-eating mammals.
Suborder Fissipedia—terrestrial Carnivora.
Family Felidae: cats—lions, tigers, etc.
Family Viverridae: civets—fossa, mongoose, meerkat, linsang, BINTURONG—ONLY SUSCEPTIBLE.
Family Hyaenidae: hyaenas.
Family Canidae: dogs—DINGO, FOX, COYOTE, WOLF—ALL SUSCEPTIBLE.
Family Procyonidae: raccoons—kinkajou, coati, bassariscus, RACCOON—ONLY SUSCEPTIBLE.
Family Mustelidae: WEASELS — FERRET, MINK, SKUNK, etc.—ALL SUSCEPTIBLE.
Family Ursidae: bears.

Susceptible species in CAPITALS; nonsusceptible in lower case letters.

In 1944, the Zoölogical Park received a young Indian mongoose (*Herpestes nyula*) which had been confiscated by the United States Customs. Since entry of mongooses into the United States is prohibited by the Fish and Wildlife Service of the Depart-

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Veterinarian of the New York Zoölogical Park, New York Zoölogical Society, New York City.

Since completing this paper, a personal communication from the Game Preservation Department, Sudan, Khartoum, reports an outbreak of feline enteritis which caused the death of two Siamese kittens, two leopard cubs, one serval cat, and one lynx. Two adult lions sickened but did not die. All deaths were limited to animals under 1 year of age. This is the first evidence we have been able to find indicating that lions are susceptible to this disease.

ment of the Interior, and the animal was to be destroyed, this was considered a good opportunity to test the susceptibility of this viverrine species to the virus of Carré. Accordingly, the animal was injected intramuscularly with 5 cc. of a 5 per cent fresh spleen suspension from a distemper dog. Simultaneously, a ferret, a member of the Mustelidae, received the same injection.

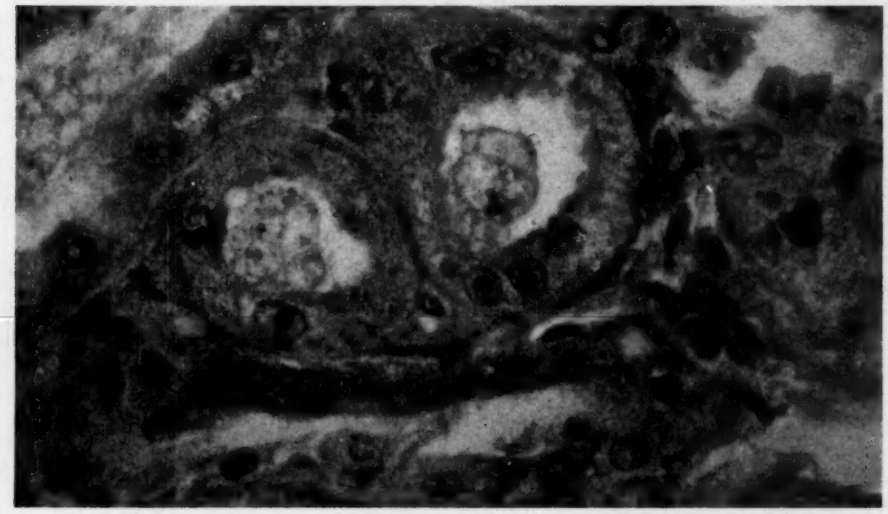

Fig. 1—Intranuclear inclusions in kidney tubules.

The mongoose remained well for five days. The ferret died in five days with typical distemper symptoms and lesions, and its tissues were heavily loaded with inclusion bodies. On autopsy, no lesions were observed in the mongoose, and no inclusion bodies could be found microscopically. Unfortunately, destruction of the mongoose was ordered at the end of the fifth day after inoculation. As a result, it can only be said that in five days the mongoose did not show any signs of infection from a virus that proved lethal for a ferret. No history was available regarding the mongoose's previous exposure to distemper, if any.

Table 1 shows that the species susceptible to canine distemper are limited to the order Carnivora and within that order to four families: the Canidae, Mustelidae, Procyonidae, and Viverridae. All of the known susceptible species fall into two of these families, namely, the Canidae and Mustelidae, except for a single member of the Procyonidae (the raccoon) and a single member of the Viverridae (the binturong).

Among the Canidae, the following are known to be susceptible: dogs, wolves, foxes, coyotes, dingoes (Australian wild dog).

In the Mustelidae, the following are known to be susceptible: skunks, mink, and ferrets. Probably all Mustelidaes are susceptible.

In the family, Procyonidae, which includes kinkajous, coatis, and bassariscus, only the raccoon is known to be susceptible.

A similar situation exists in the family Viverridae. The only member of this family known to be susceptible is the binturong. This family is composed of the civets, or catlike animals, characterized by scent glands and not completely retractile claws. They are practically restricted to African and Indo-Malayan regions with a few species in southern Europe and none naturally in North or South America. Members of this family are: the fossa, civet, mongoose, meerkat, linsang, and binturong.

We have seen canine distemper in only one member of the Viverridae family, the binturong, and have observed typical inclusion bodies in the tissues of binturongs which died after showing symptoms of canine distemper. As mentioned previously, a single attempt to produce the disease in another member of the viverrine family, the mongoose, was not successful. It is interesting to note the large number of species in the first two families (Canidae and Mustelidae) that are susceptible and the single

species susceptible in each of the other two families (Procyonidae and Viverridae).

Green claims to have produced an attenuated virus for use as an immunizing agent for domestic dogs from a remotely

TABLE 2—Feline Enteritis

Order Carnivora—flesh-eating mammals.
Suborder Fissipedia—terrestrial Carnivora.
Family Felidae: CATS. ALL SUSCEPTIBLE.
Family Viverridae: civets.
Family Hyaenidal: hyaenas.
Family Canidae: dogs.
Family Procyonidae: raccoons—kinkajou, coati, bassariscus, RACCOON—ONLY SUSCEPTIBLE.
Family Mustelidae: weasels.
Family Ursidae: bears.

Susceptible species in CAPITALS; nonsusceptible in lower case letters.

related species, the ferret. In his work, he has passed dog virus through many passages of the highly susceptible ferret. The resulting virus is considered to be relatively noninvasive for the dog.

It has been our experience, and that of others, that when outbreaks of distemper occur in collections of different species of wild animals, the virus is highly and equally pathogenic for various species. It is possible that in such outbreaks, certain species are only mildly affected and have gone unnoticed.

CAT ENTERITIS

In discussing this disease, it should first be pointed out that the nomenclature for the disease is in a confusing state. In this paper, we have reference to the disease long known as cat enteritis and recently proved by Verge and Cristoforoni,[2] Hindle and Finlay,[3] and Leasure, Lienhardt, and Taberner[4] to be caused by a filterable virus. After a virus was established as the causative agent, Lawrence and Syverton[5] and Hammon and Enders[6] studied the blood changes occurring in the disease. As a result, the disease is now variously referred to as cat enteritis, panleucopenia, agranulocytosis, cat distemper, infectious feline enteritis, etc. For the sake of uniformity and understanding, a single term for the disease should be established and adhered to. Cat plague is suggested as suitable. Panleucopenia and agranulocytosis are not considered good names because similar blood changes occur in other virus diseases, such as hog cholera.

The principal thing to be said about this disease in regard to species susceptibility is that only cats and the raccoon are susceptible. Here it is interesting to note again that the raccoon is "out of line" in being susceptible to this virus, just as he is coupled with the binturong in being susceptible to the virus of Carré. Table 2 shows that all of the members of the family, Felidae, are susceptible to the cat enteritis virus. In the family, Procyonidae, only one member (the raccoon) is susceptible to this so-called cat virus. No members of the civet family, Viverridae, are known to be susceptible. The disease has been reported in many different kinds of cats by various people, and it has been seen in practically all cats, with the exception of the lion, in

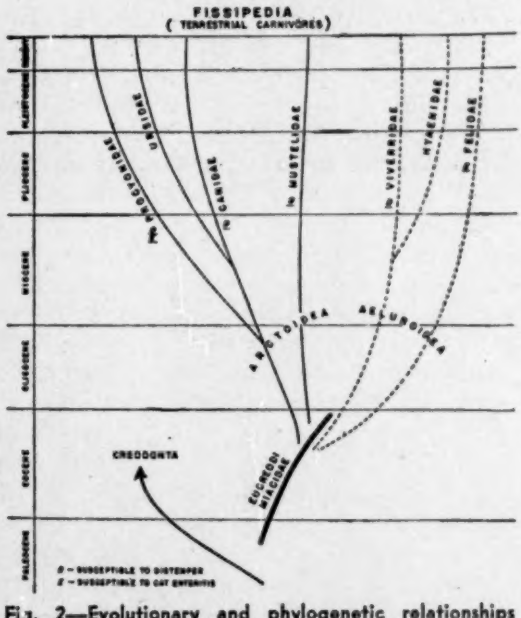

Fig. 2—Evolutionary and phylogenetic relationships among carnivora.

the New York Zoölogical Park. Undoubtedly, the lion is likewise susceptible, and probably it is just good fortune that we have not seen it in this animal. It has been seen in the raccoon by many workers and has occurred in this species in the New York

The evolutionary chart was prepared through the courtesy of Dr. Edwin H. Colbert of the American Museum of Natural History.

Zoölogical Park. Dr. Heinz A. Siedentopf has isolated the virus from raccoons.

When one attacks the matter of species susceptibility to these two viruses from an evolutionary standpoint, no reason can be found to explain the various family or species susceptibilities. Figure 2 shows the evolutionary development of the various families and also that in Eocene times, all of the terrestrial mammals (Fissipedia) had a common ancestor, the Miacidae, from which developed all the Arctoidea and Aeluroidea. Immediately after this development, the cats (Felidae) and the mink family (Mustelidae) differentiated themselves and have stayed as such up until now. As time went on, the civets (Viverridae) gave rise to the hyenas (Hyaenidae), and the dogs (Canidae) gave rise first to the raccoon family (Procyonidae) and later to the bears (Ursidae).

In the case of canine distemper, the Mustelidae, Canidae, one of the Procyonidae (the raccoon), and one of the Viverridae (the binturong) are all susceptible. With one exception, all of these had a common ancestor, the superfamily Arctoidea, back in Oligocene times. The single exception is the binturong who, strangely enough, stemmed from the Aeluroidea rather than from the Arctoidea. The bears (Ursidae) are the only descendants of the Arctoidea which are not susceptible, in spite of the fact that they are an offshoot of the Canidae, a highly susceptible group.

A similar thing is true with the feline enteritis virus. The Felidae, Hyaenidae, and Viverridae all had a common ancestor, the Aeluroidea, but of these, only the Felidae are susceptible. Of the other superfamily, Arctoidea, only one of the family, Procyonidae, (the raccoon), is susceptible to the feline virus.

From this evolutionary analysis, it is apparent that the raccoon is a unique animal in that, to date, it is the only animal proved to be susceptible to both the virus of Carré and the virus of feline enteritis.

The bear family also appears unique, in that it is the only family descending from the Arctoidea that does not have at least one species susceptible to either of these two viruses.

Further investigation may reveal other species susceptible to these viruses.

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Studies on the Diagnosis of Newcastle Disease in New Jersey

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IN 1945, we reported on the diagnosis of Newcastle disease [pneumoencephalitis] in New Jersey, and the procedure followed for isolation of the virus by egg inoculation. These studies have been continued with but minor variations in technique, and it is believed that the information gathered will be of interest to those who elect to base their diagnosis on recovery of the virus.

In the early work, tissues were collected from all cases with a symptomatology suggestive of Newcastle disease as well as from many others which were undoubtedly bronchitis, avian encephalomyelitis (epidemic tremor), or encephalomalacia. These latter were included in order to make certain that Newcastle virus was not concerned, and their inclusion naturally increased the number of negative flocks.

Brain and spleen were the tissues usually examined for virus. A few liver samples were examined, but since no virus was ever recovered from this tissue, it was assumed to be a poor source of virus, and examinations were discontinued. In the later phase of the work, yolk was collected by means of a sterile Pasteur pipette from a single ovum in laying birds, and when these samples proved fairly rich in virus the practice of collecting this tissue was continued. Likewise, all oviduct eggs were set aside for yolk collection, which was done by removing a disc of shell over the air cell and aspirating a sample of yolk with a syringe to which was attached a 2-in. 18-gauge needle.

The technique was calculated to make possible a high percentage of virus recoveries merely by the egg inoculation of sterile tissue suspensions. However, many cases that had typical histories and showed typical changes, on autopsy, failed to yield virus when sterile tissue suspensions were used for egg inoculation. Toward the end, therefore, respiratory exudates were collected for the preparation of filtrates.

Since we were interested not only in recovery of the virus, but also in the incidence of its occurrence in different organs and secretions, the question of a standard technique for filtration was considered. Lacking the time to make preliminary studies, we decided to use the Boerner centrifuge filter (2 cc.-capacity) as one likely to give reasonably consistent results. Preliminary tests with this filter brought out two serious defects. Most of the six filters used by us leaked around the screw joint, and, when used with the grids, not one gave a sterile filtrate. When both grids were removed, leakage was completely eliminated, and a contaminated filtrate was exceedingly rare.

Although several positive filtrates were obtained, the inclusion of filtrates of respiratory exudates did not materially increase the percentage of positive diagnoses. That there was considerable variation in individual filter pads was easily demonstrated by filtering 1:10, 1:100, 1:1,000, and 1:10,000 dilutions of Newcastle virus in the form of allantoic fluid through four filters, and inoculating 3 eggs with each of the filtrates. The 3 eggs receiving the 1:1,000 dilution of virus died on the third day and all the others lived through the seventh day.

In the earlier work, 0.1 cc. of tissue suspension was used for each egg, and all surviving embryos were allowed to hatch. Later, the dose per egg was increased to 0.2 cc., and incubation was continued only through the seventh day after inoculation, for the reason that no virus was ever demonstrated in eggs that died later than the sixth day after inoculation. Finally, the practice of subinoculating from embryos

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dead during the first twenty-four hours after inoculation was discontinued because, after exhaustive studies, it was found that virus could never be recovered from such embryos. It should be added that the majority of tissues were collected from cases presented at New Brunswick, since no tissues had been collected at the Vineland laboratory for approximately a year.

PERCENTAGE OF FLOCKS YIELDING VIRUS

Tissues were examined from 379 flocks. The results showed that 274 flocks failed to yield virus, whereas 105 (27.7%) were positive. From the 274 negative flocks, 572 birds were presented, or an average of 2.08 birds per flock, and from the 105 positive flocks, 283 birds were presented, or an average of 2.69 birds per flock. From the 572 birds in the 274 negative flocks, a total of 844 tissues were collected, of which 814 or 2.97 tissues per flock or 1.42 tissues per bird were examined. On the other hand, from the 283 birds in the 105 positive flocks, a total of 509 tissues were collected, of which 486 or 4.62 tissues per flock or 1.71 tissues per bird were examined. It, therefore, appears likely that virus might have been recovered more often if more birds per flock or more tissues per bird had been examined.

NEGATIVE FLOCKS

A tissue was considered negative if none of the inoculated or subinoculated embryos died within seven days after inoculation, excluding those dead within the first twenty-four hours. Since inoculations were made throughout the year, and since the quality of eggs varied with the season, it was to be expected that certain embryos would die after the first twenty-four hours and before the eighth day postinoculation. Although the changes in many of these embryos on harvest did not suggest infection, subinoculations were always carried out. The results are shown in table 1.

An analysis of the 274 negative flocks with respect to symptoms and autopsy findings was attempted, but the results were disappointing, except in a general way.

There were 63 flocks within the age limit of 1 to 4 weeks. Of these, 11 flocks showed the combination of respiratory and nervous symptoms and, excepting four flocks, also showed autopsy changes like those in Newcastle disease. Thus, at least seven of the 11 flocks were typical of Newcastle disease but failed to yield virus. In the same age group, there were 22 flocks in which the birds presented showed only respiratory symptoms, and apparently no nervous symptoms had been seen at all. Except in one flock, the autopsy findings were not unlike those of Newcastle disease. It might be argued that these latter cases were, in reality, Newcastle cases but presented too early for nervous symptoms to have developed. If this were true, then the mortality rate at the time should have been less than in flocks showing nervous symptoms. Actually, the figures available show a mortality of about 10 per cent in 17 flocks; that is, 3,382 dead in 33,800 birds from flocks showing only respiratory symptoms, and a mortality of 1,457 birds (11.8%) in five

TABLE I—Results of Tissues Examined for Newcastle Virus from 572 Birds in 274 Negative Flocks

Kind of tissue	No. collected	No. broken	No. contaminated	No. examined	Negative on — generation 1	2	3	4	5	6	7
Spleen	422	2	20	400	291	76	17	13	2	1	
Brain	265		7	258	175	50	26	3	2		2
Ovary Yolk	51		1	50	36	8	3	2	1		
Resp. Exudate	35			35	28	5	2				
Spleen & Yolk	8			8	5	2	1				
Liver	32			32	20	6	5	1			
Spleen & Brain	21			21	14	6	1				
Ovid. Egg Yolk	4			4	1	2	1				
Ovid. Tissue	2			2	1	1					
Blood Serum	4			4	3	1					
Totals	844	2	28	814	574	157	56	19	5	1	1

flocks of 12,400 birds in which both respiratory and nervous symptoms were seen.

A third classification in the 1- to 4-week age group was that in which the birds presented showed only nervous symptoms. There were 29 flocks in this category, but in 4 of these birds respiratory symptoms were reported by the owner. In only 1 case were Newcastle disease changes seen on autopsy. Obviously then, this group contained a large number of cases of avian encephalomyelitis and encephalomalacia.

The final negative flock to make the total of 63 in this age group was represented only by dead birds on which no history was available and in which air sac changes were seen.

There were 97 flocks in the 5- to 12-week age group. Twenty-three of these showed both respiratory and nervous symptoms, but seven flocks failed to show respiratory tract changes on autopsy. The 16 flocks showing typical symptoms and autopsy findings contained 19,662 birds, and at the time the cases were presented, there had been a mortality of 3,198 (16.2%).

In the same age group, specimens from 35 flocks failed to show nervous symptoms, nor were these reported by the owner. In specimens from eight of the flocks, typical autopsy changes were not found. In fact, the nasal discharge in 1 case was typical of coryza, and, in a second case, blood in the trachea was typical of laryngotracheitis. In 23 flocks in which mortality figures were available and in which the autopsy was typical, there were 34,182 birds that, when specimens were presented, showed a loss of 1,439 or only 4.2 per cent.

The last category in this age group consisted of 39 flocks in which the birds presented did not show respiratory symptoms but exhibited some type of nervous disorder. Though eight flock owners reported having seen respiratory symptoms, evidence of respiratory tract infection was seen in only 1 case. In 24 flocks on which figures are available, there were 32,346 birds, of which 1,848 (5.7%) had died at the time the cases were presented.

The third category, that of pullets, was represented by 94 flocks. The relative absence of nervous symptoms in this age group is already well known, and only five flocks showed a combination of respiratory and nervous symptoms. In 71 flocks, there were respiratory symptoms without nervous manifestations. One of these flocks was known to have coryza, and laryngotracheitis was diagnosed in two others. The birds from eight flocks showed nervous disturbances when presented for examination and in 2 of these the autopsy showed some clouding of the air sacs.

The remainder of the 94 pullet flocks, ten in number, showed neither respiratory nor nervous disturbances, and typical changes were not found on autopsy. Some of these flocks were diagnosed as having the so-called "pullet disease."

A fairly reliable guide in the diagnosis of Newcastle disease in laying birds is that of the degree of drop in egg production, a drop to about zero being highly diagnostic. On this basis, 14 flocks suffered such a drop,

TABLE 2—Results of Egg Inoculation for Newcastle Virus Isolation from Tissues of 283 Birds in 105 Flocks

Tissue	No. collected	No. contaminated	No. broken	No. examined	Positive on — Passage 1	Positive on — Passage 2	% positive	Negative on — Passage 1	2	3	4	5	7	9
Spleen	272	18		254	79	48	50.00	97	16	11	2	1		
Brain	104	1	1	102	14	6	19.60	63	11	7	1			
Mucus	65	1		64	17	3	31.25	32	11	1				
Yolk	49	2		47	14	6	42.55	17	5	3	2			
Ovid. Egg	10			10	2	1	30.00	3	2	1	1			
Liver	2			2			.00						1	1
Serum	1			1			.00	1						
Pool	6			6	2	2	66.66	2						
Total	509	22	1	486	128	66	39.91	215	45	23	6	1	1	1

and 35 flocks suffered a loss of 50 per cent or more. In 18 flocks, the loss was less than 50 per cent. In 27 instances, the effect on egg production was not known.

A miscellaneous group of 20 flocks, to make a total of 274, was composed of four flocks of hens, five of turkeys, two of pigeons, one blackbird, and a miscellany of eight in the form of exudates from birds in flocks on which there was no history of symptoms, age, or mortality.

lem, the positive tissues are arranged with reference to age groups in table 3.

Table 3 shows that, in general, and in those cases in which an appreciable number of tissues was examined, the virus is more easily recovered from younger birds than from more mature ones. Thus, the virus was recovered from 62.82 per cent of the spleens from chicks 1 to 4 weeks old, from 59.79 per cent of those 5 to 8 weeks old, from 37.5 per cent of the few examined in

TABLE 3—Distribution of Virus in Various Tissues with Reference to Age

	1—4 Weeks		5—8 Weeks		9—16 Weeks		Pullets		Hens		Turkeys		Total	
Tissue	No.	% +	No.	% +	No.	% +	No.	% +	No.	% +	No.	% +	No.	% +
Spleen	78	62.82	97	59.79	8	37.50	70	24.28	1	.00			254	50.00
Brain	34	29.41	31	22.59	2	50.00	34	2.94	1	100.00			102	19.60
Mucus	20	30.00	9	33.33			34	32.35	1	.00			64	31.25
Liver			2	.00									2	.00
Yolk							46	43.47	1	.00			47	42.55
Serum			1	.00									1	.00
Ovid. Egg							10	30.00					10	30.00
Pool			2	100.00			2	50.00			2	50.00	6	66.66
Total	132	49.24	142	49.29	10	40.00	196	27.05	4	25.00	2	50.00	486	39.91

POSITIVE FLOCKS

As already noted, the virus was recovered from 105 flocks from which 283 birds were presented for examination. A total of 509 tissues was collected with the results of egg inoculations listed in table 2.

Table 2 shows that, of the 486 tissues examined, 194 (39.91%) yielded the virus. It is also apparent that, of the tissues examined, the virus shows an unequal distribution, being most consistently present in the spleen, somewhat less so in ovarian yolk, and still less in the yolk of oviduct eggs and filtrates of respiratory exudates. The percentage of positive respiratory exudate filtrates would probably increase materially if all virus could be recovered by filtration.

Although only 39.91 per cent of the tissues examined yielded the virus, the percentage of positive birds was greater, since more than a single tissue was examined from many birds. Thus, in 262 birds one or more tissues were examined, and of these the tissues from 166 (63.35%) were positive.

It is generally recognized that the virus is most easily recovered from young birds. In order to throw some light on the prob-

the 9- to 16-week age group, and from only 24.28 per cent of the pullets. The same decrease in incidence of virus recovery with increase in age is seen in the case of the brain, where appreciable numbers were examined. Occurrence of virus in respiratory exudates did not appear to decrease with increase of age, and a possible explanation may be that much more exudate can be collected from the larger bird.

It is thought by some that virus is more readily recovered from the living bird than from the dead one. To get some information on this point, the 262 birds on which results are available were arranged with reference to their status when presented for examination. The results are found in table 4.

The results set forth in table 4 show that virus was recovered at least as readily from dead as from living birds, regardless of the age of the bird.

BLIND PASSAGES

Table 2 shows that of the 194 virus isolations, 128 were recovered in primary inoculation in embryonating eggs. That is to say, every embryo died within the six days post-inoculation. All subinoculations thereafter, except from an embryo dead within twenty-

four hours after inoculation, have resulted in 100 per cent embryo mortality. However, in 66 isolations, 1 or more primarily inoculated embryos failed to die, but on the first subinoculation from 1 of the dead embryos, the 100 per cent mortality was obtained with one exception, which will be described below, and in which 100 per cent mortality was not obtained until the third passage. In other words, with the exception just mentioned, we have never recovered virus after the second passage when subinoculations were made with material from dead embryos, even when as many as nine passages were made.

Our findings are at variance with those of Beach[2], who reported that in many cases several passages had to be made before the embryos died and that, in one instance, embryos did not die until the thirty-second passage. Unfortunately, Beach did not report the postinoculation time at which material was collected for subinoculation, nor did he say specifically what tissue was used. Presumably, material from live embryos was used. Since Beach has reported that embryos die of Newcastle infection in twenty-four to ninety-six hours, it may be assumed that material for subinoculation was collected not later than ninety-six hours On several occasions we have recovered virus from an embryo dead on the fifth or sixth day in cases in which this was the only embryo to die on primary passage. In our studies, subinoculations were always made with a suspension of liver from a dead embryo, except in the attempts to recover virus by blind passage as described below.

In these somewhat limited studies, cases were selected in which, on primary inoculation, 1 or more embryos survived at least seven days after inoculation, and in which 1 or more embryos died within six days and yielded a liver which, on subinoculation, killed 100 per cent of the embryos. In such a case, in which Newcastle infection was definitely established in the dead embryo, it seemed that if it were possible for virus to exist in other eggs similarly inoculated for a period of seven days, these should serve as the most suitable starting point in a study concerned with the necessity of making blind passages. In these studies, the one or more surviving embryos of the first passage supplied inoculum for the next passage. Instead of the liver of the embryo the allantoic fluid was used because the inoculation had been by this route. It was reasoned that the virus would be more likely to exist here than in the liver, because to reach the liver would require generalization and therefore death.

The cases studied in this manner are set forth in table 5. In the first case listed, it will be seen that the splenic suspension was inoculated into 3 embryonating eggs with the result that 2 died in four days and the third egg was still alive on the seventh day. A subinoculation with a liver suspension of 1 of the four-day dead embryos into 3 eggs three days later resulted in the death of these in three, two, and two days, respectively. The changes in these eggs were typical for Newcastle disease, and the eggs were shown to be bacteriologically sterile. Allantoic fluid was collected from the 1 egg that survived seven days and was inoculated sixteen days later into 3 eggs, of which 1 died on the third day, and 2 survived the seventh day. Fluid was recovered from each of the latter eggs, pooled, and thirty-nine days later was inoculated into 4 eggs, all of which survived seven days postinoculation. The 1 embryo which died on the third day in the second passage supplied a liver suspension that, on inoculation forty-three days later, killed all 4 embryos, but only 1 after the twenty-four-hour period. The liver from the latter supplied a suspension which, on inoculation five days later, permitted survival of 2 embryos through seven days, death of a third on the seventh day, and death of a fourth on the second day. A suspension of the liver of the latter embryo on inoculation five days later did not kill any of the 3 embryos within seven days. Thus, while the virus was demonstrated in the 1 egg that died on primary inoculation, it was not possible to recover it from an egg surviving seven days even though four more serial passages were made.

Here attention must be directed to the large number of embryo deaths within twenty-four hours. Unfortunately, these tests were carried out during a period when the quality of hatching eggs was very poor.

Table 5 shows that, with one exception, virus could not be recovered by subinoculations from live embryos. The exception concerns eggs

TABLE 4—Incidence of Virus in Dead and Living Birds with Reference to Age

Age Group	Dead			Alive		
	No.	+	% +	No.	+	% +
1 - 4 weeks	22	16	72.72	57	37	64.91
5 - 8 weeks	15	11	73.73	83	53	63.85
9 -16 weeks				8	4	50.00
Pullets	3	2	66.66	71	41	57.74
Hens				1	1	100.00
Turkeys				2	1	50.00
Total	40	29	72.50	222	137	61.71

inoculated with S spleen 3 suspension, in which 1 embryo died in two days, and 2 survived seven days in the primary inoculation. On subinoculation from the two-day dead embryo made thirty-two days later the 3 embryos died within twenty-four hours. Ordinarily, a reinoculation would have been made, but as the inoculum had been inadvertently discarded, virus was not actually demonstrated in this case. However, a pool of the allantoic fluids from the 2 surviving eggs, when inoculated fifty-one days later, killed all 4 embryos, 1 in twenty-four hours, and 3 in two days. The changes in the embryos and yolk sacs were typical, and the material was shown to be bacteriologically sterile.

Table 5 also shows the only exception, in our experience, to the rule that in all Newcastle virus isolations a mortality of 100 per cent was attained by the second passage and maintained thereafter. The exception concerns eggs inoculated with a pool of spleens in the K case. On primary passage, 1 embryo survived seven days, and a subinoculation from it, seven days later, failed to kill any of the 4 embryos inoculated. The other 2 eggs, on primary inoculation, died on the second day. One of these supplied a liver suspension that, eight days later, permitted 1 of the 3 embryos inoculated to survive seven days, but killed the other 2 in one and two days, respectively. The third and fourth passages made at intervals of fourteen and six days resulted in 100 per cent embryo mortality.

SYMPTOMS AND AUTOPSY FINDINGS IN POSITIVE BIRDS

The symptoms reported by the owners or seen in the birds presented for examination in flocks from which virus was recovered were typical for Newcastle disease. In young birds these included respiratory symptoms alone or in combination with nervous manifestations. Autopsy of young birds showed cloudy air sacs with occasional small amounts of exudate and bronchial plugs and some mucus in the trachea. Occasionally, small pieces of caseous exudate occurred in the trachea. Pullet flocks rarely showed nervous symptoms, but respiratory symptoms were reported in every flock from which virus was recovered. Egg production was reported to be markedly depressed within a few days and, in many instances, fell almost to zero. There was also the history of a marked increase in

TABLE 5—Attempts to Recover Virus from Live Embryos in Lots in Which a Dead Embryo Yielded Virus

	1st Passage	I	2nd Passage	I	3rd Passage	I	4th Passage	I	5th Passage	Virus Recovered
	D4 (D4)	3	D3 D2 D2							×
W. Sp.1		16	(D3)	43	D1 D1 D1 (D2)	5	A7 A7 D7 (D2)	5	A7 A7 A7	—
	(A7)	16	(A7 A7)	39	A7 A7 A7 A7					—
	(D3)	7	D4 D2 D2							×
E. Yolk 2	(A7)	8	A7 A7 A7							—
	(A7)	57	A7 D1 D1 (D3)	4	A7 A7 (D6)	1	A7 A7 D1 D1			—
	(D4)	30	D1 D1 (D2)	5	D2 D2 D2					×
S. Sp.1	(A8)	47	A7 A7 (D6)	1	A7 A7 A7 D1					—
	(A8)	47	A7 D1 D1							—
S.	(D2)	32	D1 D1 D1							?
Sp. 3	(A7 A7)	51	D1 D2 D2 D2							×
	(D3)	21	D1 D1 (D3)	11	D2 D2 D2					×
Ro. Sp. 1	(A8)	45	D1 D1 A7							—
	(A8)	45	D1 A7 A7							—
W.	(D3)	32	D1 D1 (D2)	11	D1 D1 (D2)	4	D2 D2 D2 D2			×
Muc. 1	(A8 A8)	49	D1 A7 A7 A7							—
S&S.	(D3)	15	D1 D3 D3							×
Muc. 3	A7 (A7)	28	D1 D1 A7							—
H.	D2 (D3)	15	D1 D1 (D2)	5	D3 D2 D2					×
Muc. 1	(A7)	28	D1 A7 A7							—
	(D6)	15	D1 D2 D3							×
H. Br. 2	(A7)	28	D1 A7 A7							—
	(A7)	32	D1 D1 A7							—
	(D3)	15	D2 D2 D3							×
H. Muc. 2	(A7)	28	D1 D1 A7							—
	(A7)	28	D1 D1 A7							—
Ru.	(D3)	11	D1 D1 (D2)	5	D2 D2					×
Yolk 2	D1 (A8)	23	D1 D1 A7							—
K.	D2 (D2)	8	D1 A7 (D2)	14	D1 D3 (D2)	6	D2 D2 D2			×
Pool Sp.	(A7)	7	A7 A7 A7 A7							—

I = interval between passages in days; A7 = alive on seventh day post injection; D3 = dead on third day post injection; symbol in parenthesis indicates egg used for passage.

the number of floor and roost eggs during the drop in production, and many had soft shells or were without shells. On resumption of production, shell color often showed changes, that is, heavy breeds produced white eggs. The autopsy findings invariably showed clouding of the air sacs. Caseous plugs in the bronchi did not occur, but the trachea contained mucus and occasionally flakes of caseous exudate. The ova contained thin yolk and were flabby.

In relation to symptoms, the virus was recovered more often from flocks in which only respiratory symptoms were reported. Thus, in the 1- to 4-week old group, 25 chicks from flocks showing both respiratory and nervous symptoms were examined, and the virus was recovered from 12 or 48 per cent, whereas it was recovered from 30 (76.92%) of the 39 chicks from flocks reporting only respiratory symptoms. In the 5- to 8-week old group of 35 chicks from flocks whose owners reported both respiratory and nervous symptoms, 19 or 54.28 per cent yielded virus. On the other hand, virus was recovered from 44 or 72.13 per cent of 61 chicks from flocks whose owners reported only respiratory symptoms.

GEOGRAPHIC DISTRIBUTION OF POSITIVE FLOCKS

Of the 105 flocks from which virus has been isolated, 102 were in New Jersey, 1 in Delaware, and 2 in Pennsylvania. The 102 New Jersey flocks were distributed by counties as follows: Atlantic 2, Bergen 3, Burlington 2, Camden 1, Cumberland 11, Essex 1, Gloucester 2, Hunterdon 2, Mercer 2, Middlesex 8, Monmouth 19, Morris 1, Ocean 36, Passaic 1, Salem 1, Somerset 6, Sussex 1, and Union 3. The figures are not an accurate index of the relative distribution of the disease in the state, except that Monmouth and Ocean Counties were severely stricken and rank first and third, respectively, in poultry population. Cumberland County also has a dense poultry population, ranking second in the state, but, as already reported, no tissues have been examined from this area for approximately a year. The nearness of Monmouth and Ocean Counties to New Brunswick also contributed to the greater number of specimens presented and hence isolations.

Only three counties in the state have failed to yield positive cases, *viz.*, Cape May, Hudson, and Warren. These counties rank sixteenth, twenty-first, and thirteenth, respectively, in poultry population.

SUMMARY

1) From 379 flocks, most of which showed symptoms suggestive of Newcastle disease, 855 birds were examined. Two hundred and seventy-four flocks from which 572 birds were examined, an average of 2.08 birds per flock, failed to yield virus. One hundred five flocks (27.7%) from which 283 birds were examined, an average of 2.69 birds per flock, yielded virus. From the 572 birds in negative flocks 844 tissues were collected of which 814 were suitable for examination. This is an average of 2.97 tissues per flock or 1.42 tissues per bird. On the other hand, from the 283 birds in positive flocks 509 tissues were collected of which 486 were suitable for examination: an average of 4.62 tissues per flock or 1.71 tissues per bird. It, therefore, seems likely that if a larger number of birds per flock or a larger number of tissues per bird had been examined the percentage of positives would have increased.

2) Of the 814 negative tissues, 574 were determined as such on primary inoculation in eggs, 157 had to be carried to the second generation, 56 to the third, 19 to the fourth, five to the fifth, one to the sixth, and two to the seventh.

3) Of the 486 tissues from positive flocks, 194 (39.91%) yielded the virus. Of these, 128 were determined as positive on primary inoculation in eggs and 66 on the second passage. Of the 292 negative tissues from positive flocks, 215 were determined as such on primary passage, 45 had to be carried to the second passage, 23 to the third, six to the fourth, and one each to the fifth, seventh and ninth. The virus showed an unequal distribution in different tissues, occurring in 50 per cent of the spleens, 42.55 per cent of ovarian yolks, 31.25 per cent of the respiratory exudate filtrates, 30 per cent of oviduct egg yolks, 19.6 per cent

of the brains, and in 66.66 per cent of pooled samples.

4) Although only 39.91 per cent of the tissues from positive flocks were positive, the number of positive birds was 166 or 63.35 per cent of the 262 on which one or more samples were examined.

5) In general, the virus was more readily recovered from younger birds. Contrasting extreme age groups, for example, the virus was recovered from 62.82 per cent of 78 spleens from chicks 1 to 4 weeks old, and from only 24.28 per cent of 70 pullet spleens. Again, the virus was recovered from 29.41 per cent of 34 brains from chicks 1 to 4 weeks old, but from only 2.94 per cent of 34 pullet brains. The percentage of positive respiratory exudate filtrates did not seem to decrease when collected from older birds, but this may be due to the larger amount of recoverable exudate in older birds.

6) Virus was as readily recovered from dead birds as from living ones, regardless of age, being recovered from 72.5 per cent of 40 dead birds, and from 61.71 per cent of 222 living birds.

7) Attempts to recover virus from embryos that had lived through seven days postinoculation on primary inoculation were successful only once, and in this case, all embryos of the second passage died. In the 12 attempts, the embryos that died on primary passage, with one possible exception, yielded virus.

8) The virus has been recovered from birds in 18 of the 21 counties in New Jersey, from one flock in Delaware, and from two in Pennsylvania.

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The Effect of Sulfaquinoxaline Medication on Eimeria Tenella Infection in Chickens

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SINCE THE discovery of sulfanilamide and its successful application in clinical medicine, a whole galaxy of sulfonamide drugs has appeared, some of which have proved superior to the parent drug. Among the newest of these arrivals is sulfaquinoxaline, first reported by Weijlard, Tischler, and Erickson[1] in 1944. The first to demonstrate the potential usefulness of this drug in veterinary medicine was Delaplane[2] who, in 1945, showed 0.05 and 0.1 per cent in the mash to be effective prophylactically against chronic fowl cholera. In a further report Delaplane, Batchelder, and Higgins[3] found the same drug concentrations to be effective both prophylactically and therapeutically against cecal coccidiosis in the chicken. These levels of drug intake, far below those known to be effective clinically for any other commonly known sulfonamide drug, indicate that sulfaquinoxaline perhaps may find an eminent position among products useful in poultry disease control.

EXPERIMENTAL

Toxicity Trials.—Toxicity for the host at therapeutic levels is of primary importance in the study of any new product for disease control. In order to check the toxicity of sulfaquinoxaline for the chick, 210 newly hatched, White Leghorn cockerels were divided into five lots of 42 each. Two lots were fed 0.1 per cent sulfaquinoxaline in the mash for ten and thirty days, respectively; two lots were fed 0.3 per cent drug in the mash for the same periods of time, while the fifth group served as controls. The chicks were weighed at the beginning of the trials and thereafter every five days for thirty days. The results are shown in graph 1. It is seen that 0.1 per cent sulfaquinoxaline in the mash up to thirty days depressed growth rate, but was not associated with important mortality. However, 0.3 per cent of the drug in the mash severely depressed growth rate and definitely is within the toxic range, since 9 of the 42 birds so treated for thirty days died, and many of the survivors were badly stunted. It is noteworthy, however, that normal growth was promptly resumed upon withdrawal of the drug after ten days at both levels of intake.

Activity of Sulfaquinoxaline Against Cecal Coccidiosis.—The toxicity trials being encouraging, clinical testing was begun against cecal coccidiosis in the chicken. In an effort to locate the minimum effective level of drug intake per day, groups of 4- to 6-week-old White Leghorn cockerels were infected—each bird with 15,000 sporulated oöcysts of *Eimeria tenella* and subsequently treated with ½-, 1-, and 2-gr. single doses of sulfaquinoxaline per pound of body weight per day for varying intervals following infection. For this purpose, the drug was dissolved in sodium carbonate solution and pipetted directly into the crops of the birds. Mortality at this exposure level was negligible. The results are shown in table 1.

From table 1, it is seen that sulfaquinoxaline medication at the level of ½ gr. per pound of body weight per day for varying two- and three-day intervals following infection was ineffective in suppressing the development of cecal coccidiosis. On the other hand, a drug intake of 2 gr. per pound of body weight per day for any two- or three-day period after infection, up to and including the sixth 24-hour period, appeared to suppress completely the progress of the disease. The effect of medication at the 1-gr. level per pound of body weight per day was intermediate between the two extremes.

Mention should be made of drug action upon oöcyst sporulation. At the level of ½ or 1 gr. of drug intake per pound of body weight per day, up to and including the fourth day after infection, there was little if any influence upon the viability of oöcysts shed on the seventh day. In all cases, however, when the birds were treated on the fifth day either at the ½- or 1-gr. intake level, great damage was occasioned to the oöcysts eliminated on the seventh day. Most of the bodies were grossly abnormal, showing vacuolations and capsule distortions, while less than 1 per cent of them were observed to sporulate after seventy-two hours in 1.5 per cent dichromate solution. Ripson

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Appreciation is expressed to the medical department of Merck & Co., Inc., Rahway, New Jersey, for supplying the sulfaquinoxaline used in these studies.

and Herrick[4] have reported sulfadiazine to exert similar action.

The above results indicated the minimum effective therapeutic level of sulfaquinoxaline against cecal coccidiosis to be near 1 gr. per pound of body weight per day. It was desired next to determine the minimum treatment period necessary for maximum benefit. A trial, the details of which are not shown, indicated single-day treatments of 1 gr. of drug per pound of body weight to be unsatisfactory since marked hemorrhage occurred in all birds so treated.

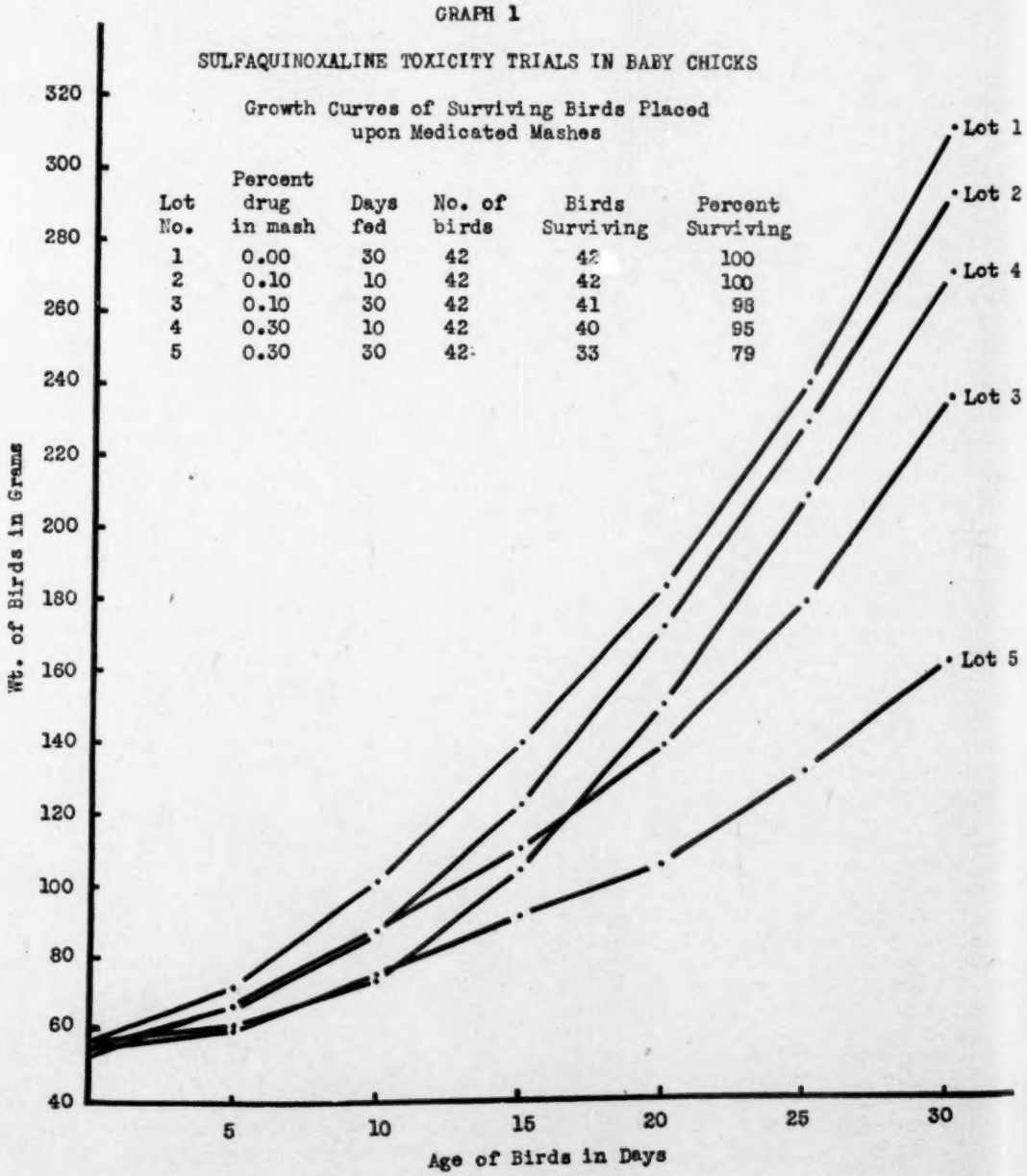
By measuring the feed consumption of a group of birds, a drug intake of 1 gr. per pound of body weight per day was calculated to correspond closely to the drug intake when administered at the 0.1 per cent level in the mash. In order to determine the relative effectiveness of two- and three-day treatment periods at the 0.1 per cent drug intake level in the mash and to check this against a drug intake of 0.2 per cent in the mash for two-day periods, 216, 6- to 8-week-old White Leghorn cockerels were divided into 18 groups for growth curve and mortality studies. Each in-

GRAPH 1

SULFAQUINOXALINE TOXICITY TRIALS IN BABY CHICKS

Growth Curves of Surviving Birds Placed upon Medicated Mashes

Lot No.	Percent drug in mash	Days fed	No. of birds	Birds Surviving	Percent Surviving
1	0.00	30	42	42	100
2	0.10	10	42	42	100
3	0.10	30	42	41	98
4	0.30	10	42	40	95
5	0.30	30	42	33	79



fected bird receive 75,000 sporulated oöcysts of *Eimeria tenella*. Results are shown in graphs 2 and 3 and in table 2. Curves on the graphs represent average growth rates of birds surviving the coccidial infections. Lot numbers indicated in table 2 correspond to lot numbers appearing on graphs 2 and 3.

appeared in lot 8, 1 bird out of 12 succumbing as compared with a 75 per cent loss in the controls.

In graph 3, sulfaquinoxaline medication at the 0.2 per cent level in the mash, while of benefit, is shown to be in the toxic range. The administration of the drug itself at this intake

TABLE I—Prophylactic and Therapeutic Activity of Sulfaquinoxaline Against Cecal Coccidiosis When Administered in Single, Daily Oral Dosages to 4- and 6-Week-Old Chicks

No. of chicks*	Sporulated oöcysts per bird	Drug admin. per bird per day (grains/lb. body wt.)			Days treated**	Postmortem, 7th day					
						Hemorrhage			Oöcysts		
						Drug admin.			Drug admin.		
		0.5	1.0	2.0		0.5	1.0	2.0	0.5	1.0	2.0
12	15,000	0.5	1.0	2.0	1, 2	+	+	—	+	+	—
12	15,000		Same		2, 3	+	+	—	+	+	—
12	15,000		"		3, 4	+	—	—	+	+	—
12	15,000		"		4, 5	+	+	+	+	+	—
12	15,000		"		5, 6	+	+	+	+	±	—
12	15,000	0.5	1.0	2.0	1, 2, 3	+	+	—	+	+	—
12	15,000		Same		2, 3, 4	+	+	—	+	+	—
12	15,000		"		3, 4, 5	+	—	—	+	—	—
12	15,000		"		4, 5, 6	+	+	+	+	—	—

*Drug administered at each level to four chicks in the various groups.
**Days treated following exposure to coccidia. 1 day refers to treatment simultaneously with exposure. 2 day refers to treatment twenty-four hours after exposure, and so forth.

In graph 2 is shown the result of sulfaquinoxaline medication at the 0.1 per cent intake level in the feed for varying two- and three-day intervals, following exposure of the birds to coccidiosis. Lots 1 to 5 represent the bird groups infected with coccidia and treated with 0.1 per cent drug in the mash for two-day periods after infection but at different intervals. Lots 6 to 9 indicate the bird groups treated for three-day periods following infection, while lots 10, 11, and 12 represent the controls, one infected, one drug-treated, and one unmolested group.

It should be noted that the feeding of 0.1 per cent sulfaquinoxaline in the mash for three days had no apparent influence upon growth rate. The superiority of medication for three-day over two-day periods is readily seen, for while three of the growth curves (lots 1, 4, 5) for groups treated for two-day periods broke sharply on the fifth and sixth days, only one growth curve (lot 6) for bird groups treated for three-day periods indicated appreciable growth disturbance during the same period of time.

The survivors of all infected groups, together with the birds in control lot 11, were reëxposed to 75,000 sporulated oöcysts of *Eimeria tenella* on the tenth day in order to test the degree of immunization. Although many birds subsequently passed blood, all previously exposed groups were shown to be markedly immune, for only the birds in lot 8, treated at the three-, four-, and five-day interval, showed appreciable growth retardation. In table 2, it is seen that the only mortality occurring in previously infected groups

level was characterized by sharp distortions in the growth curves. Each surviving bird showed high resistance to a challenging disease exposure on the tenth day of 75,000 sporulated oöcysts.

In table 2 are shown the mortality figures and the percentage weight changes occurring during the periods of hemorrhage for all lots represented in graphs 2 and 3. Referring to the column, "Wt., change, 4th to 7th days," it is seen that of the three-day medicated groups only lot 6, referred to above, and treated during the one-, two-, three-day interval following exposure showed a significant growth deviation during the hemorrhage period as compared with the control group, lot 11. All bird groups medicated with sulfaquinoxaline at the 0.1 per cent level in the mash for two-day periods showed either actual weight loss or a slower growth rate during the bleeding period as contrasted with the controls. Of those groups medicated at the 0.2 per cent level in the mash for two-day periods, only lot 15 showed a growth response during the fourth to seventh day interval comparable with the controls. Referring to graph 3, it is seen that the weight change occurring in this group for the indicated period probably was influenced by drug action. Referring to lots 10 and 11, table 2, it is seen that sulfaquinoxaline medication at the 0.1 per cent intake level in the mash for three days did not adversely affect growth rate. This conclusion is further substantiated by a study of growth curves 6, 7, 8, and 9 in graph 2.

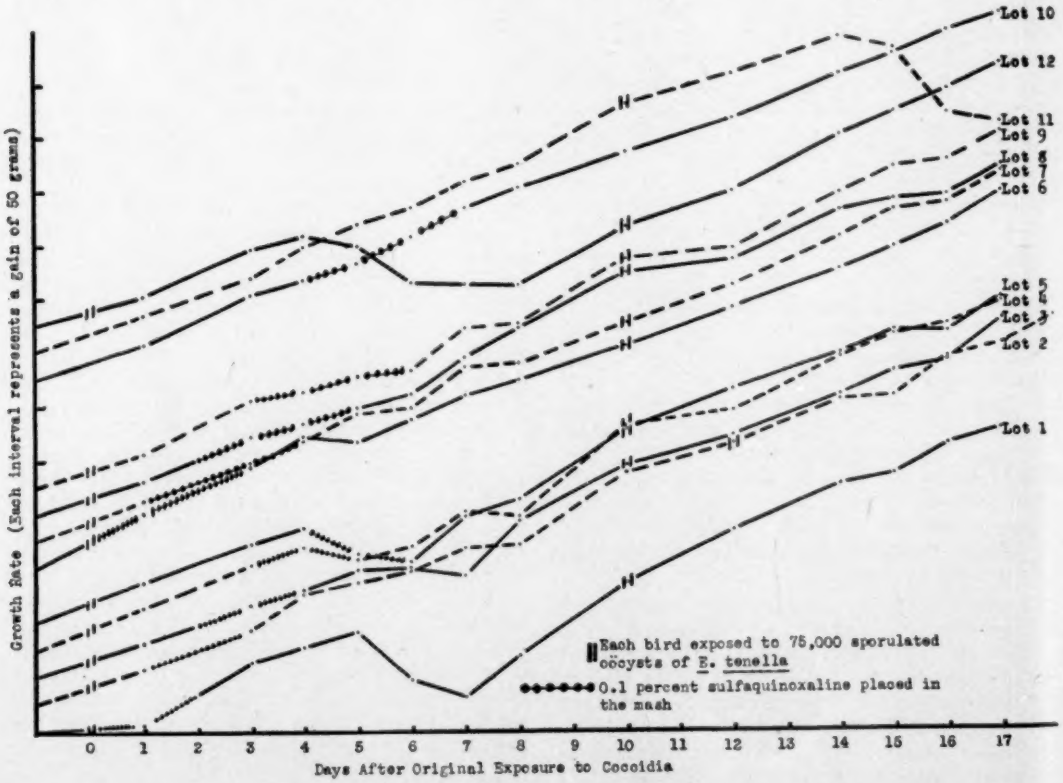
While the differences are not great and may not be highly significant considering the small

number of birds, the mortality record presented in table 2 and associated with the original exposure to coccidia is in favor of the three-day as compared with the two-day medicated groups.

Sulfaquinoxaline Medication Through the Drinking Water.—It was desired next to check the relative effectiveness of sulfaquinoxaline medication through the medium of drinking water as compared with feed. By measuring the feed and water consumption of a group of birds, the drug intake per day at a level of 0.04 per cent in the water was estimated to approximate closely the drug intake per day at the 0.1 per cent level in the mash. For the trials, 238, 4-week-old White Leghorn cockerels were divided into 14 groups for growth curve and mortality studies. Each infected bird was exposed to 50,000 sporulated oöcysts of *Eimeria tenella.* Medication was for two-day periods at intervals of one and two days, four and five days, and five and six days, after infection. Three groups were given 0.1 per cent sulfaquinoxaline in the mash for the indicated periods while corresponding groups were treated with 0.03, 0.04, and 0.05 percentages respectively of the drug in the drinking water. Two control groups, a drug-treated lot at the 0.04 per cent level in the water for three days, and an unmolested lot, were maintained.

The results are shown in graph 4 and table 3. Lot numbers indicated in graph 4 correspond to lot numbers appearing in tabble 3. Referring to graph 4, growth rate differentials occurring between the fourth and seventh days probably do not indicate a significant difference between sulfaquinoxaline medication at the 0.1 per cent level in the feed as compared with the 0.04 or 0.05 percentages in the water. The growth curves, however, of groups receiving 0.03 per cent sulfaquinoxaline in the water (lots 1, 5, 9) broke sharply on the fifth and sixth days, indicating this degree of medication to be sub-optimal.

It is noteworthy that a drug intake of 0.04 per cent in the water for three days did not, in itself, appreciably affect growth rate (lots 13 and 14, graph 4). There was some disturbance, however, for of the 17 birds in the drug-treated group, 6 showed slight weight decline during the first twenty-four hours,


Growth Rate (Each interval represents a gain of 50 grams)
Lot 10
Lot 12
Lot 11
Lot 9
Lot 8
Lot 7
Lot 6
Lot 5
Lot 4
Lot 3
Lot 2
Lot 1
Each bird exposed to 75,000 sporulated oöcysts of E. tenella
0.1 percent sulfaquinoxaline placed in the mash
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Days After Original Exposure to Coccidia

Graph 2—Growth curves of bird groups, each bird in the infected lots being exposed to 75,000 sporulated oöcysts of Eimeria tenella and the groups subsequently placed upon mash containing 0.1 per cent sulfaquinoxaline for two- and three-day periods. A second exposure to coccidia was made on the tenth day in order to measure immunization resulting from the first exposure.

whereupon normal growth apparently was resumed.

Referring to mortality figures in table 3, sulfaquinoxaline administration through the water at either the 0.04 or 0.05 per cent level is indicated to be superior to medication through the mash at the 0.1 per cent level. Of the total birds treated through the mash, 22 (42.3%) succumbed to coccidiosis as compared with 11 and 13 (21.6% and 26.0%, respectively) of those medicated at the 0.04 and 0.05 per cent drug levels in the water. Of the birds receiving 0.03 per cent sulfaquinoxaline in the water, 23 (45.1%) died, indicating again this degree of medication to be sub-optimal.

DISCUSSION

In the control of coccidiosis by the use of medicinal agents, maximum efficiency will be achieved when, by the use of the minimum amount of material for the shortest period of time, the following will be accomplished: (1) quick reduction of death losses to negligible proportions, (2) obviation of weight loss or severe growth depression in birds passing through the disease, (3) development of a state of immunity to further infections. In addition, the drug itself should be nontoxic at therapeutic levels.

Sulfaquinoxaline, when administered at a level of 0.1 per cent of an all-mash ration or 0.04 per cent of the drinking water for a period of three days, may closely approximate these specifications. The margin of safety, however, is not a wide one. A lowered intake level may not accomplish the most complete control while higher intakes quickly encounter toxic ranges.

It should be pointed out, however, that the birds used in arriving at these values were very heavily exposed to coccidia, since 75 per cent or more of the exposed controls died. Natural outbreaks of cecal coccidiosis seldom run this virulent course, and experience may show sulfaquinoxaline medication for intervals less than three days to be sufficient for satisfactory control of many field outbreaks. Probably, however, 0.1 per cent drug in an all-mash ration or 0.04 per cent in the drinking water represent near minimum daily intakes for maximum therapeutic benefit.

The work reported herein suggests the administration of sulfaquinoxaline through the drinking water to be superior to administration through the feed. In table 3, greatest differences are seen to occur in those groups treated during the fourth and fifth 24-hour periods after infection. Possibly the more favorable effect of medication through the water is due to a more

TABLE 2—Prophylactic and Therapeutic Activity of Sulfaquinoxaline When Administered in the Mash Against Infection with Eimeria Tenella

Lot No.	Birds	Sporulated oöcysts 0 day	Drug conc. in mash (%)	Days fed*	Wt. change 4th to 7th days (%)	Mortality No.	Mortality (%)	Sporulated oöcysts 10th day	Wt. change 14th to 17th days (%)	Mortality No.	Mortality (%)
1	12	75,000	0.1	1, 2	— 5.9	1	8.3	75,000	5.3	0	
2	12	75,000	0.1	2, 3	5.7	0		75,000		0	
3	12	75,000	0.1	3, 4	2.2	0		75,000	7.6	0	
4	12	75,000	0.1	4, 5	5.8	3	25.0	75,000	5.6	0	
5	12	75,000	0.1	5, 6	1.6	5	41.7	75,000	5.4	0	
6	12	75,000	0.1	1, 2, 3	5.2	0		75,000	8.0	0	
7	12	75,000	0.1	2, 3, 4	10.3	0		75,000	7.3	0	
8	12	75,000	0.1	3, 4, 5	10.4	0		75,000	6.2	1	8.3
9	12	75,000	0.1	4, 5, 6	9.4	2	16.7	75,000	7.0	0	
13	12	75,000	0.2	1, 2	— 1.0	1	8.3	75,000	7.3	0	
14	12	75,000	0.2	2, 3	5.7	0		75,000	8.8	0	
15	12	75,000	0.2	3, 4	13.5	0		75,000	5.6	0	
16	12	75,000	0.2	4, 5	5.7	1	8.3	75,000	5.8	0	
17	12	75,000	0.2	5, 6	— 0.9	3	25.0	75,000	5.9	0	
10	12	0	0.1	3 days	11.9**	0		0	7.4	0	
11	12	0	0.0		10.2	0		75,000	—5.5	9	75.0
12	18	75,000	0.0		— 7.8	14	77.7	75,000	9.8	0	
18	12	0	0.2	2 days	0.0**	0					

*Days fed following exposure to coccidia. 1 day refers to the first 24-hour period following exposure. 2 day the second twenty-four hours, and so forth.

**The percentage weight changes are for the days during which the drug was administered, being three and two days, respectively.

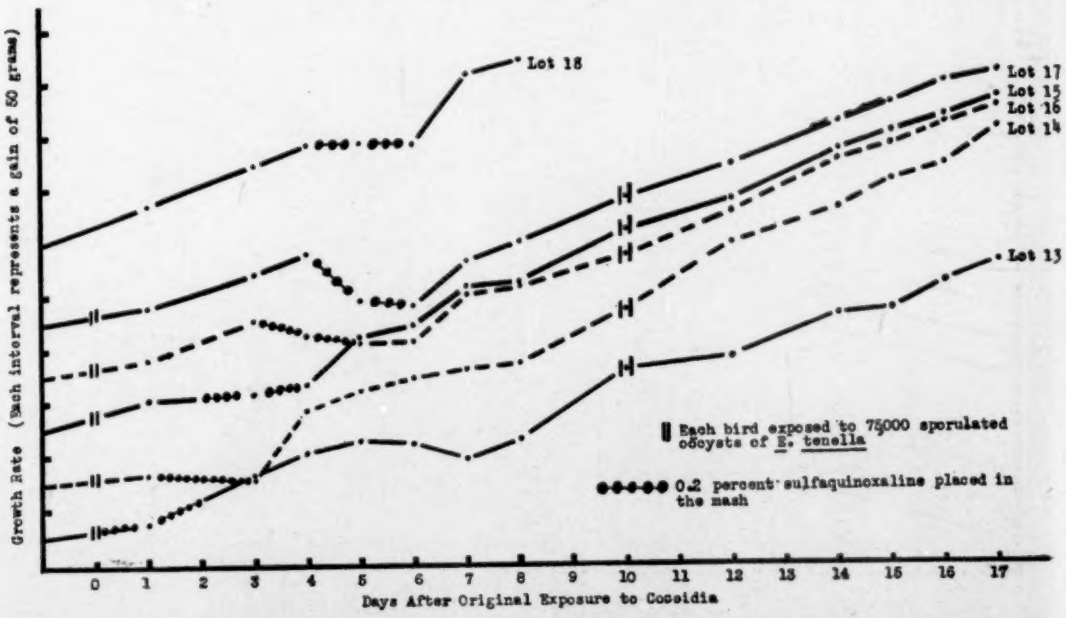
rapid establishment of an effective drug level in the blood stream. By the beginning of the fourth day, the development of cecal coccidiosis is well advanced, and the second generation merozoites will shortly erupt, initiating the critical period of hemorrhage. Time is running out, and, if the progress of the disease is to be interrupted, or significantly moderated, an effective blood concentration of the therapeutic agent must be established within the shortest possible time. With sulfaquinoxaline, this perhaps may be accomplished more quickly by administering the drug in the drinking water rather than in the feed. Cameron and McOmie[5] found sulfaquinoxaline to be absorbed by sheep more rapidly when administered as a drench than in capsules.

TABLE 3—Relative Prophylactic and Therapeutic Activity of Sulfaquinoxaline Against Cecal Coccidiosis When Administered in the Mash and in the Drinking Water

Lot No.	Birds	Sporulated oöcysts per bird	Drug conc. in feed or water	Treatment period (days)*	Mortality	
					No.	Per cent
1	17	50,000	0.03% water	1, 2	9	53.0
2	17	50,000	0.04% water	1, 2	2	11.8
3	17	50,000	0.05% water	1, 2	4	23.5
4	18	50,000	0.10% feed	1, 2	5	27.8
5	17	50,000	0.03% water	4, 5	5	29.4
6	17	50,000	0.04% water	4, 5	1	5.9
7	17	50,000	0.05% water	4, 5	0	0.0
8	17	50,000	0.10% feed	4, 5	4	23.5
9	17	50,000	0.03% water	5, 6	9	53.0
10	17	50,000	0.04% water	5, 6	8	47.0
11	16	50,000	0.05% water	5, 6	9	56.2
12	17	50,000	0.10% feed	5, 6	13	76.4
13	17	0	0.04% water	3	0	
14	17	0	0.00		0	

*Treatment period following exposure to coccidia. 1 day refers to the first 24-hour period following exposure, 2 day the second 24-hour period, and so forth.

Sulfaquinoxaline appears to be effective against every stage in the life cycle of

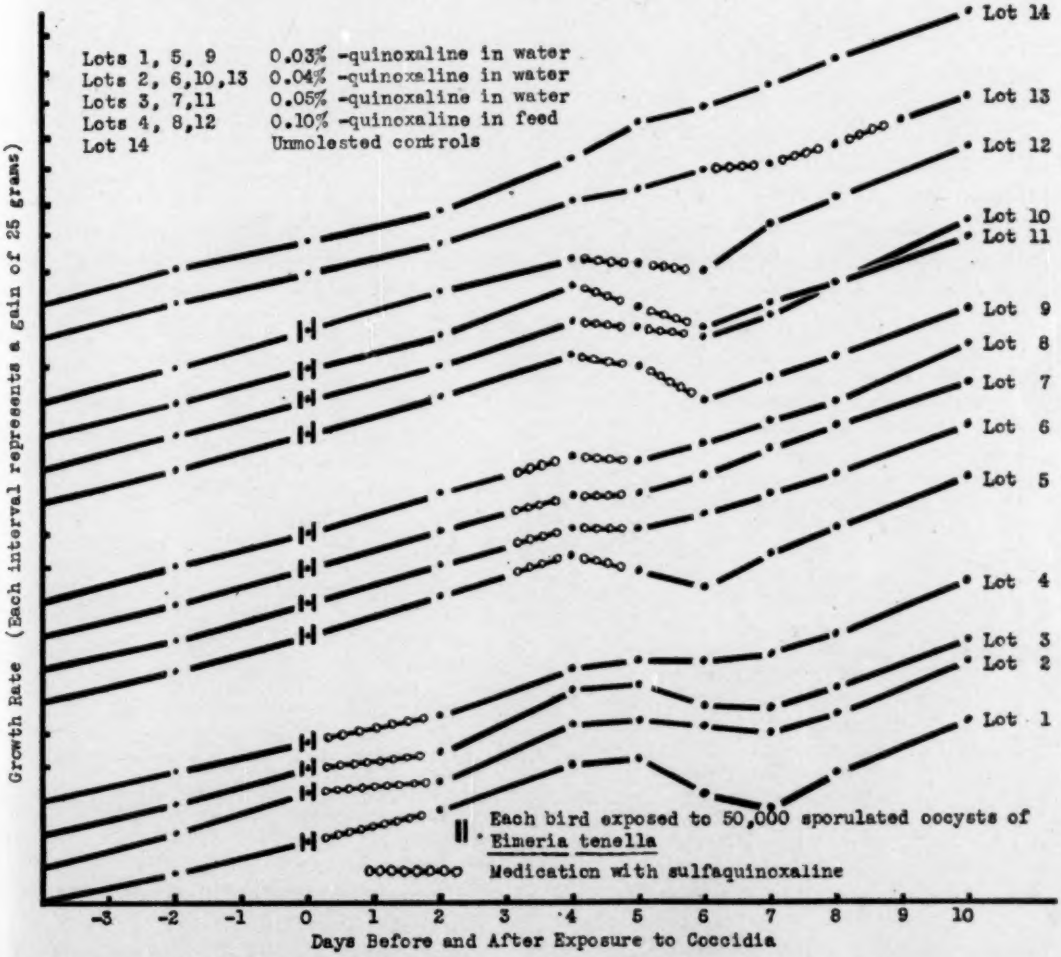

Growth Rate (Each interval represents a gain of 50 grams)
Lot 18
Lot 17
Lot 15
Lot 16
Lot 14
Lot 13
Each bird exposed to 75000 sporulated oöcysts of E. tenella
0.2 percent sulfaquinoxaline placed in the mash
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Days After Original Exposure to Coccidia

Graph 3—Growth curves of bird groups, each bird in the infected lots being exposed to 75,000 sporulated oöcysts of Eimeria tenella, and the groups subsequently placed upon mash containing 0.2 per cent sulfaquinoxaline for two-day periods. A second exposure to coccidia was made on the tenth day in order to measure immunization resulting from the first exposure.

Eimeria tenella, although at different minimal concentrations. Histologic studies, however, will be necessary for precise evaluations. With an exposure of 15,000 sporulated oöcysts, ½ gr. of drug per pound of body weight per bird per day when administered upon the fifth day was sufficient to inhibit normal sporogony, since the vast majority of the oöcysts eliminated upon the seventh day were nonviable. Twice this amount of drug administered during the three-, four-, five-day interval was necessary in order to suppress the development of the disease, while 2 gr. of drug per pound of body weight per bird per day were necessary to inhibit the course of the disease during the first forty-eight hours. Ripson and Herrick[4] found sulfadiazine to vary also in its effectiveness against different stages in the life cycle of *Eimeria tenella*.

CONCLUSIONS

1) Sulfaquinoxaline is an effective therapeutic agent against cecal coccidiosis in the chicken. For maximum therapeutic benefit, the minimum drug intake per bird is estimated to be near 1 gr. per pound of body weight per day. For the birds used in the trials, this intake was calculated to correspond closely to the drug intake when administered as 0.1 per cent of an all-mash ration or as 0.04 per cent in the drinking water.


Lots 1, 5, 9 0.03% -quinoxaline in water
Lots 2, 6,10,13 0.04% -quinoxaline in water
Lots 3, 7,11 0.05% -quinoxaline in water
Lots 4, 8,12 0.10% -quinoxaline in feed
Lot 14 Unmolested controls
Lot 14
Lot 13
Lot 12
Lot 10
Lot 11
Lot 9
Lot 8
Lot 7
Lot 6
Lot 5
Lot 4
Lot 3
Lot 2
Lot 1
Each bird exposed to 50,000 sporulated oocysts of Eimeria tenella
Medication with sulfaquinoxaline
Growth Rate (Each interval represents a gain of 25 grams)
-3 -2 -1 0 1 2 3 4 5 6 7 8 9 10
Days Before and After Exposure to Coccidia

Graph 4—Growth curves of bird groups, each bird in the infected lots being exposed to 50,000 sporulated oöcysts of Eimeria tenella, and the groups subsequently placed upon sulfaquinoxaline medicated mash or water for two-day periods.

2) Sulfaquinoxaline when administered in therapeutic levels at any time up to seventy-two hours after heavy exposure of birds to cecal coccidiosis is able, markedly, to moderate the clinical manifestations of the disease. Treatment after ninety-six hours, while of benefit, is able only in part to avert the consequences of the disease after heavy exposure.

3) It is postulated that near maximum efficiency will be achieved in the use of sulfaquinoxaline for the control of cecal coccidiosis after heavy exposures of the birds when the drug is administered in the drinking water at a concentration of 0.04 per cent for three days, or as 0.1 per cent of an all-mash ration for the same period of time.

4) The administration of sulfaquinoxaline in the drinking water was found to be superior to administration through the feed. The effect was most marked in those birds treated during the 72- to 120-hour interval after infection.

5) Administration of sulfaquinoxaline to baby chicks at the 0.1 per cent level in the mash for thirty days considerably depressed growth rate but was not associated with important mortality. Administration of the drug at the same level for three days to 4- to 8-week-old chicks did not affect growth rate. Sulfaquinoxaline administered to chicks at intake levels of 0.2 per cent or more in the mash was toxic as manifested by severe growth depression.

6) Sulfaquinoxaline appears to be effective against every stage in the life cycle of *Eimeria tenella*, although at different minimal concentrations. Further studies will be necessary for precise evaluations.

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Some Pharmacologic Aspects of Sulfaguanidine in Normal Chickens

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THE INVESTIGATION of the effect of sulfaguanidine on normal chickens, reported herein, was undertaken to furnish information concerning safe therapeutic dosages of the drug for cecal coccidiosis. It was also considered of interest to determine if the drug is as readily absorbed from the intestinal tract of chickens as had been shown by Marshall *et al.*[1] and Bankowski[2] for ducks and turkeys.

The specific points investigated were: (a) the rate of absorption of sulfaguanidine, (b) the maximum nontoxic doses tolerated by chickens, and (c) the concentration of sulfaguanidine in the blood and intestine following administration of varying amounts of sulfaguanidine.

MATERIALS AND METHODS

All experimental chickens were single-comb White Leghorn males and females, obtained as day-old chicks from a local hatchery. They were reared in battery brooders and fed an all-mash ration which, through several years' usage, had been found to provide ample nourishment.

Determinations of Sulfaguanidine Concentrations.—All concentrations, unless otherwise mentioned, were determined as free sulfaguanidine.

The concentrations of the drug in the blood were determined by the method of Marshall and Bratton,[3] using a photoelectric colorimeter for measuring the color reactions. The blood specimens were drawn from the wing vein into a syringe containing powdered potassium oxalate, and the determinations were made with 0.5-cc. samples of blood, using trichloracetic acid for protein precipitation.

Material for determination of the sulfaguanidine concentration of the intestine was obtained by sacrificing the chickens and by collecting the contents of the desired portions of the intestinal tract. The determinations were made according to the method of Marshall *et al.*[4]

*Single Doses of Sulfaguanidine.**—To study the effect of sulfaguanidine administered in a single dose, a group of chickens was given the drug orally in hard gelatin capsules. The doses were as follows: 6 chickens, 64 days old, were given 0.5 Gm. per kilogram of body weight; 6, 39 days old, 1 Gm. per kilogram of body weight; 5, 72 days old, 2 Gm. per kilogram of body weight; and 6, 135 days old, 3 Gm. per kilogram of body weight. The birds were under constant observation for symptoms, and blood samples were taken at frequent intervals during the daylight hours to determine the concentration of the drug in the blood.

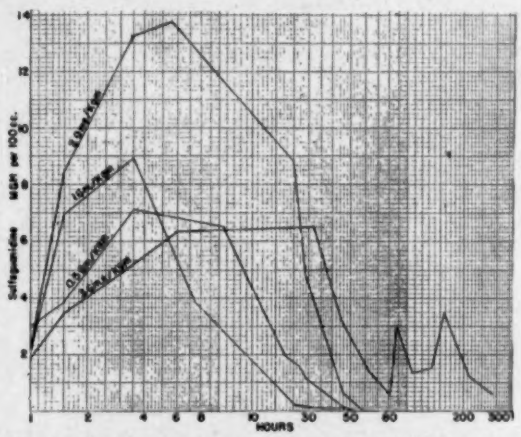

Fig. 1—Blood-concentration time curves following the oral administration of 0.5, 1.0, 2.0, and 3.0 Gm. of sulfaguanidine per kilogram. Each curve represents the average of the curves of 5 or 6 individual chickens.

Sulfaguanidine Administered with the Feed.—Investigation was made of the effect of sulfaguanidine on chickens, and of concentrations of the drug in the intestinal contents and blood, following its administration mixed with the feed. It was felt that this would be the most practical way of giving it on farms for the control of coccidiosis.

Three groups of 36 chickens, 60, 55, and 70 days old, were starved for a period of twelve hours and then fed continuously mash contain-

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*The sulfaguanidine used in the experiment reported herein was supplied by the Lederle Laboratories, Pearl River, New York.

ing, respectively, 0.5 per cent, 1.0 per cent, and 2.0 per cent sulfaguanidine. Four chickens of each group were sacrificed on the second, sixth, twelfth, twenty-fourth, thirty-sixth, forty-eighth, fifty-fourth, seventy-second, and seventy-eighth hours, and the sulfaguanidine concentrations in the blood, cecums, and anterior and posterior halves of the intestine were determined. The results are shown in tables 1, 2, and 3.

RESULTS AND DISCUSSION

Effects of Sulfaguanidine Administered in a Single Dose.—The rate of absorption of sulfaguanidine, as indicated by the time required to reach the maximum concentration in the blood, was markedly variable in individual birds and did not appear to be correlated with the amount of drug administered (fig. 1). In the chickens given 0.5 Gm. per kilogram of body weight, the time before the peak concentration was reached varied from one and a half to ten and a half hours; in the 6 chickens which had received 1 Gm. per kilogram of body weight, from one and a half to three and a half hours; and in the 5 which were given 2 Gm. per

TABLE 1—Sulfaguanidine concentrations in the blood, anterior half, posterior half, and cecums of 36 chickens, 61 days old, given 0.5 per cent sulfaguanidine mash for seventy-eight hours.

Bird Number	Sulfaguanidine Concentration: mgm/100 cc Blood	mgm per 100 grams: Anterior Half	mgm per 100 grams: Posterior Half	mgm per 100 grams: Ceca	Time Sacrificed
1	0.90	48.00	25.60	25.30	2 Hours
2	1.20	26.00	44.00	4.00	10:30 AM
3	1.20	32.00	72.00	0.00	
4	0.80	6.00	64.00	1.60	
Average	1.05	28.00	51.40	9.65	
5	2.40	14.00	102.00	378.00	6 Hours
6	3.00	51.00	66.00	102.00	2:30 PM
7	2.20	72.00	44.00	98.00	
8	3.00	70.00	118.00	338.00	
Average	2.65	51.75	82.50	229.00	
9	3.00	26.00	26.00	397.00	12 Hours
10	2.60	26.00	16.00	80.00	8:30 AM
11	2.00	1.60	25.00	268.00	
12	1.60	8.00	12.00	285.00	
Average	2.30	15.40	19.75	257.50	
13	3.20	23.00	51.00	718.00	24 Hours
14	2.20	26.00	40.00	365.00	8:30 AM
15	1.60	32.00	28.00	512.00	
16	3.20	12.00	48.00	432.00	
Average	2.55	23.25	41.75	506.75	
17	4.50	40.00	56.00	232.00	36 Hours
18	3.20	67.00	56.00	192.00	8:30 PM
19	3.20	26.00	38.00	120.00	
20	1.80	26.00	80.00	204.00	
Average	3.17	39.25	57.50	212.00	
21	1.40	14.00	-	100.00	48 Hours
22	1.00	2.00	32.00	660.00	8:30 AM
23	0.50	0.00	60.00	472.00	
24	0.70	0.00	14.00	728.00	
Average	0.90	4.00	35.33	490.00	
25	5.10	23.00	104.00	512.00	54 Hours
26	4.30	44.00	142.00	376.00	2:30 PM
27	4.30	76.00	120.00	552.00	
28	5.60	87.00	107.00	416.00	
Average	4.82	57.50	118.25	464.00	
29	3.40	19.00	124.00	400.00	72 Hours
30	2.20	32.00	85.00	400.00	8:30 AM
31	3.00	12.00	58.00	348.00	
32	2.60	10.00	66.00	348.00	
Average	2.80	18.25	83.25	374.00	
33	3.80	24.00	96.00	760.00	78 Hours
34	3.00	26.00	76.00	272.00	2:30 PM
35	3.40	38.00	108.00	400.00	
36	3.40	44.00	216.00	416.00	
Average	3.40	33.00	124.00	462.00	
Mean	2.60	30.10	69.10	333.60	
Std. Dev.	1.31	22.24	42.54	200.68	

kilogram of body weight, from one and a half to twenty-four hours. The peak concentration in 13 of the 17 chickens (77%) occurred within three and a half hours after the drug was administered. The average peak concentrations found in the blood, however, varied in accordance with the size of the dose, being 6.76, 8.31, and 15.88 mg. per 100 cc. of blood, respectively, for the groups given 0.5 Gm., 1.0 Gm., and 2.0 Gm. per kilogram of body weight. The data obtained on the absorption of sulfaguanidine by the 6 chickens given 3 Gm. per kilogram of body weight were unsatisfactory, because of the impossibility of securing blood samples for study between the sixth and thirty-second hours after the drug had been administered. Nevertheless, the rate of absorption was considerably slower since the blood concentrations on the sixth

TABLE 2—Sulfaguanidine concentrations in the blood, anterior half, posterior half, and cecums of 36 chickens, 55 days old, given 1 per cent sulfaguanidine in the mash for seventy-eight hours.

Bird Number	Sulfaguanidine Concentration: mgm/100cc Blood	mgm per 100 grams: Anterior Half	Posterior Half	Ceca	Time Sacrificed
1	1.80	32.00	208.00	56.00	2 Hours
2	3.40	40.00	166.40	72.00	10:30 AM
3	2.40	48.00	80.00	80.00	
4	4.30	69.60	201.60	23.20	
Average	2.97	47.40	164.00	57.00	
5	7.60	64.00	119.20	680.00	6 Hours
6	8.00	80.00	185.60	680.00	2:30 PM
7	10.90	72.00	262.40	337.60	
8	9.10	38.40	192.00	432.00	
Average	8.90	63.60	189.80	532.40	
9	6.80	38.40	119.20	348.00	12 Hours
10	7.90	38.40	33.40	640.00	8:30 AM
11	8.40	19.20	33.40	512.00	
12	6.00	19.00	-	-	
Average	7.27	28.75	62.00	500.00	
13	4.00	19.00	6.40	1340.00	24 Hours
14	3.60	23.00	23.20	1072.00	8:30 AM
15	3.60	19.00	1.60	1424.00	
16	6.40	38.00	8.00	984.00	
Average	4.40	24.75	9.80	1205.00	
17	7.20	12.00	132.00	864.00	36 Hours
18	6.40	48.00	72.00	489.60	8:30 PM
19	7.40	19.20	72.00	680.00	
20	7.40	25.60	19.20	19.20	
Average	7.10	26.20	73.80	513.20	
21	5.50	19.20	88.00	1688.00	48 Hours
22	6.70	40.00	148.00	1392.00	8:30 AM
23	5.80	56.40	48.00	1240.00	
24	6.90	88.00	136.00	192.00	
Average	6.22	50.90	105.00	1128.00	
25	5.80	106.00	140.00	1140.00	54 Hours
26	4.30	88.00	128.00	1566.00	2:30 PM
27	9.10	152.00	192.00	1620.00	
28	6.70	70.00	98.40	1620.00	
Average	6.47	104.00	139.60	1486.50	
29	8.00	48.00	64.00	1480.00	72 Hours
30	6.40	48.00	96.00	1168.00	8:30 AM
31	8.00	80.00	80.00	904.00	
32	10.60	144.00	152.00	1592.00	
Average	8.25	80.00	98.00	1286.00	
33	6.40	19.20	54.40	600.00	78 Hours
34	8.20	25.60	56.00	1520.00	2:30 PM
35	4.00	16.00	40.00	1456.00	
36	4.50	4.00	28.80	600.00	
Average	5.77	16.20	44.80	969.00	
Mean	6.4	49.00	104.50	875.40	
Std.Dev.	2.11	34.49	68.55	526.00	

hour did not approach the values obtained with the smaller doses. The toxicity of the drug, which was evident two hours following administration of the sulfaguanidine, may have been the cause for the slow rate of absorption. Litchfield[5] reported incomplete and erratic rates of absorption of sulfanilimide following large doses.

The rate of elimination of sulfaguanidine from the blood was fairly rapid and uniform following single doses of 0.5 Gm., 1.0 Gm., and 2.0 Gm. per kilogram of body weight, and, by the fifty-eighth hour, no trace could be detected in the blood of any of the chickens of the three groups. It was slow and irregular, however, in the birds given 3 Gm. per kilogram of body weight, and small amounts were present in the blood on the 287th hour, the time of the last test. A chemical analysis of the cecal contents of each of these chickens still revealed the presence of 4.8 to 10.9 mg. of sulfaguanidine per 100 Gm. of cecal contents.

No toxic manifestation was noted in chickens which were given 0.5 Gm. of the drug per kilogram of body weight. Depression and anorexia were the only symptoms observed in birds which received 1 and 2 Gm. of the drug per kilogram of body weight. With the former dosage, the depression was slight and was noted only between the twenty-fourth and thirty-second hours following administration. With the latter dosage (2 Gm.), these symptoms were notably present five hours later and were unabated for twenty-four hours, when the conditions of the birds gradually improved. All appeared normal by the eighty-eighth hour, the time the birds were sacrificed. Two hours following the administration of 3 Gm. of the drug per kilogram of body weight, anorexia, marked depression, staggering gait, and tendency to sit were noted. This was followed, at about the sixth hour, by a circulatory failure as evidenced by paleness of the skin and mucous membranes and difficulty in securing blood by venepuncture. One bird also showed cyanosis from the thirty-second to the sixty-third hour; the other 5 were considerably improved by the forty-fourth hour, and all 6 appeared normal on the sixty-sixth hour. One, however, had a relapse, manifested by cyanosis from the 109th to the 133rd hour.

Effect of Administering Sulfaguanidine in the Feed.—Sulfaguanidine, incorporated in the feed and fed *ad libitum*, was found to be applicable to flock treatment. In chickens given 0.5, 1.0, and 2 per cent sulfaguanidine mashes, the relative trend of changes in the concentrations in the blood and in the three levels of the intestinal tract was similar, whereas the absolute amounts of the drug varied in these organs according to its content in the feed. The amount of sulfaguanidine found varied among individual chickens fed the same mash; however, the variations were not so great as some of those following single doses. The concentrations of the drug in the blood and in the three portions of the intestine are given in tables 1, 2, and 3.

The concentration of the drug in the blood fluctuated, as would be expected, with the time of day when the samples were taken for the chemical analysis. Two to six hours after the feeding was begun, a rapid rise of the drug in the blood was found. On the twelfth hour, which ran into the evening of the same day, a slight decrease in the concentration was noticed. Generally, however, samples taken in the afternoon or evening contained higher values for sulfaguanidine than those which were taken in the morning. The average concentrations on the twenty-fourth and forty-eighth hours, when the blood was obtained in the morning, were 1.72 mg. per 100 cc., with 0.5 per cent sulfaguanidine mash; 5.30 mg. per 100 cc. with the 1 per cent sulfaguanidine mash; and 11.62 mg. per 100 cc. on the 2.0 per cent sulfaguanidine mash. The average concentrations in the blood during the afternoon and evening periods were 3.26 mg. per 100 cc., 7.16 mg. per 100 cc., and 14.20 mg. per 100 cc. of blood, respectively. Since sufficient mash was kept in the hoppers at all times, the values for the morning sulfaguanidine concentrations included the drug absorbed from the feed consumed before 8:30 a. m., when the blood samples were taken.

The average sulfaguanidine concentrations in the intestinal tract were found to

be lowest in the anterior half, higher in the posterior half, and greatest in the cecums. The average concentrations of the drug in the upper and lower portions of the intestine, as in the blood, fluctuated during the course of the experiment, being lowest in the morning, highest in the afternoon, and intermediate in the evening hours. Although the fluctuations in the average concentrations between the anterior and posterior levels of the intestine paralleled each other, this relationship between the cecums and the intestine was not present. In the cecum, less fluctuation was noted at different times of the day, and the trends of the variations which occurred were independent of those for the anterior and posterior intestines.

It can be reasonably assumed, therefore, that sulfaguanidine is accumulated in the

TABLE 3—Sulfaguanidine concentrations in the blood, anterior half, posterior half, and cecums of 36 chickens, 70 days old, given 2 per cent sulfaguanidine in the mash for seventy-eight hours.

Bird Number	Sulfaguanidine Concentration				Time Sarificed
	mgm/100cc	mgm per 100 grams			
	Blood	Anterior Half	Posterior Half	Ceca	
1	7.40	60.00	106.40	40.00	2 Hours
2	5.80	90.40	337.60	80.00	10:30 AM
3	6.10	76.00	469.60	272.00	
4	8.50	54.40	116.80	72.00	
Average	6.95	70.20	257.60	116.00	
5	9.10	72.00	174.40	472.00	6 Hours
6	9.90	72.00	122.40	288.00	2:30 PM
7	11.30	54.40	218.40	232.00	
8	10.60	69.60	192.00	480.00	
Average	10.22	67.00	176.80	368.00	
9	13.70	80.00	196.80	544.00	12 Hours
10	15.00	25.60	94.40	288.00	8:30 AM
11	10.60	64.00	128.00	544.00	
12	7.40	23.20	102.40	832.00	
Average	11.65	48.20	130.15	552.00	
13	7.20	54.40	110.40	900.00	24 Hours
14	12.60	66.40	196.80	1040.00	8:30 AM
15	18.30	116.80	770.00	872.00	
16	13.30	80.00	365.60	584.00	
Average	12.85	79.40	360.20	849.00	
17	13.30	40.00	25.60	1024.00	36 Hours
18	21.10	160.40	381.60	284.00	8:30 PM
19	24.90	80.00	352.00	904.00	
20	15.90	32.00	102.40	1592.00	
Average	18.80	64.60	215.40	976.00	
21	15.90	64.00	185.60	1456.00	48 Hours
22	7.60	76.00	192.00	2480.00	8:30 AM
23	-	14.40	56.00	336.00	
24	7.60	60.00	119.20	1664.00	
Average	10.36	53.60	138.20	1484.00	
25	-	64.00	192.00	1104.00	54 Hours
26	19.40	102.40	218.40	904.00	2:30 PM
27	-	98.40	302.40	1592.00	
28	22.00	64.00	255.20	832.00	
Average	20.70	82.20	242.00	1108.00	
29	14.60	33.60	98.40	944.00	72 Hours
30	11.30	69.60	337.60	544.00	8:30 AM
31	2.80	76.00	83.20	696.00	
32	2.00	38.40	28.80	400.00	
Average	7.67	54.40	137.00	646.00	
33	15.90	80.00	180.00	1064.00	78 Hours
34	14.20	102.40	337.60	1280.00	2:30 PM
35	24.90	98.40	415.20	560.00	
36	24.90	98.40	317.60	1104.00	
Average	19.97	94.80	317.60	1002.00	
Mean	12.87	68.26	219.52	788.94	
Std.Dev.	6.21	24.63	144.74	519.69	

cecums. Brown[6] has demonstrated that the proportion of the ingesta entering the cecums varied directly with the fluidity of the contents of the rectum and that only fluids entered. Olson and Mann[7] have also demonstrated that water-soluble dyes and carbon in colloidal form gained entrance into the cecums but that lamp-black did not gain entrance in appreciable amounts. Sulfaguanidine apparently entered the cecums in solution and probably to some degree in an undissolved form. The continual entrance of fecal matter containing the drug, together with the great rate of reabsorption of fluids from the cecums and rectum, resulted in its high concentration.

Sulfaguanidine was found in the cecums in small amounts two hours after feeding was begun and in appreciable amounts by the sixth and twelfth hours. In all cases, the concentration of drug in the cecums between the twelfth and twenty-fourth hours reached a value close to the seventy-eighth hour mean; however, the concentrations varied in individual chickens. These variations can be expected, due to the normal periodic emptying of these organs. Similar variations with water-soluble dyes and colloidal carbon were reported by Olson and Mann.[7]

Chickens which were fed the 1 per cent and 2 per cent sulfaguanidine mashes eliminated an abundance of visible sulfaguanidine in the form of white powder admixed with the cecal contents. Analysis of the latter showed very little difference in sulfaguanidine concentrations among chickens which were fed these two levels. This was unexpected in view of the fact that the sulfaguanidine values in the blood and in the anterior and posterior halves of the intestine varied with the amount of drug administered. Furthermore, previous studies with chickens of approximately the same age, fed a mash containing 2 per cent sulfaguanidine, reported an average sulfaguanidine concentration of about 2,000 mg. per 100 Gm. of cecal content. No satisfactory explanation can be given for the low sulfaguanidine concentrations in the present experiment.

No acute toxic manifestations were noted in chickens given a mash containing 0.5 per cent, 1.0 per cent or 2.0 per cent sulfaguanidine, and fed *ad libitum* for a period of seventy-eight hours. Moreover, no pathologic lesions were found upon autopsy of the chickens which were given the drug in the feed or in single doses.

Acetylation of Sulfaguanidine.—There was no evidence to indicate that sulfaguanidine is appreciably acetylated in the body of the chicken. Free and total sulfaguanidine concentrations were made upon many occasions, with no significant differences between the unhydrolyzed and hydrolyzed filtrates. Two chickens were placed on a 2.0 per cent sulfaguanidine mash for a period of ten days and then sacrificed. The kidneys were removed and subjected to a chemical analysis for free sulfaguanidine and its acetyl derivative. None of the latter was found; however, the kidneys contained 80 and 18 mg. of free sulfaguanidine per 100 Gm. of tissue, respectively.

SUMMARY

1) The rate of absorption of sulfaguanidine from the gastrointestinal tract of chickens which were given single doses of 0.5 Gm., 1.0 Gm., and 2.0 Gm. of the drug (in capsules) per kilogram of body weight was markedly variable in individual birds; however, the average peak concentrations in the blood were proportional to the amount of drug administered. The rate of absorption and elimination of a larger dose (3 Gm./1 Kg.) was considerably slower. One, 2, or 3 Gm. of sulfaguanidine per kilogram given as single doses were toxic.

2) The absorption of sulfaguanidine following its administration *ad libitum* in the feed was more complete and proportional to its concentration in the mash. Sulfaguanidine fed at the rate of 0.5 per cent, 1.0 per cent, and 2.0 per cent for a period of seventy-eight hours did not produce symptoms of acute toxicity.

3) Chickens fed sulfaguanidine *ad libitum* in mash showed a constant and consistently higher concentration in the cecum than in the anterior or posterior intestinal tract. In the latter portions of the intestine,

as well as in the blood, the concentrations of the drug fluctuated considerably and were related to the feeding habits of chickens, being lowest in the morning, highest in the afternoon, and intermediate in the dark, evening hours.

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The Gross- and Micropathology of Sulfaquinoxaline Poisoning in Chickens

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DELAPLANE[1] reported the efficacy of sulfaquinoxaline given in the mash of six experimental groups of young broiler chickens in preventing upper respiratory infection of birds inoculated with field material containing *Pasteurella avicida*. In another experiment to study the efficiency of a mash containing 0.05 per cent sulfaquinoxaline in overcoming *P. avicida* infection, laying birds were housed under conditions simulating those on farms. Symptoms of occult poisoning in some birds became evident approximately eight days after starting the medicated feed. This was in contrast to the response of young broiler birds previously reported, in which no such symptoms were observed during medicated feeding periods ranging up to two weeks, and on levels of 0.05 and 0.1 per cent in the feed.

The gross lesions consisting of whitish foci in the spleen, liver, kidneys, lungs, and heart were similar to those observed by Farr and Wehr,[2] Thorp,[3] and Levine[4] as occurring with sulfonamides in chickens. Granulomatous lesions in man produced by other sulfonamides have been reported by More, McMillan, and Duff,[5] and French.[6] Recently, Farr and Jacquette[7] described lesions as a result of sulfamerazine medication of chickens and the influence of age on the prevalence of the lesions.

The purpose of this paper is to record the findings in birds which died as a result of sulfaquinoxaline toxicity.

MATERIALS AND METHODS

Three laying houses, 20 ft. by 20 ft., at the Division of Veterinary Science experimental farm housed the Rhode Island Red pullets used in the studies. The houses were designated as follows: Pen 1 consisted of 86 pullets which were exposed to enzoötic *P. avicida* infection by placing a laboratory infected bird in contact with them on Sept. 8, 1945; pen 2 consisted of 86 pullets also exposed by introducing a laboratory infected bird as a pen contact on the same date. These birds were given 0.05 per cent sulfaquinoxaline in the mash. Pen 3 consisted of 99 pullets somewhat older and constituted the normal control to determine any great differences in egg production traceable to either the *P. avicida* infection or the sulfaquinoxaline feed.

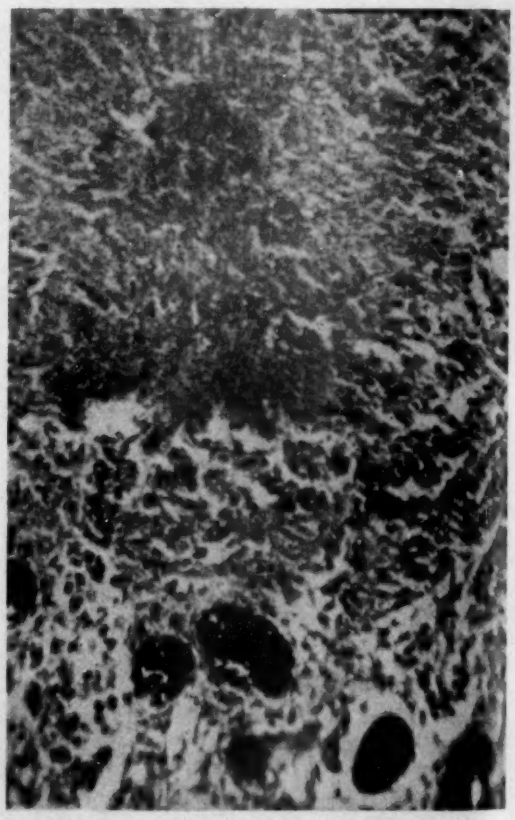

Fig. 1—Lung-caseation in lung nodule. × 175.

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Poultry pathologist (Delaplane), Division of Veterinary Science, Texas Agricultural Experiment Station, Agricultural & Mechanical College of Texas, College Station, Texas; resigned November 30, 1945. Formerly veterinary pathologist (Milliff), now head of Department of Veterinary Anatomy, Agricultural & Mechanical College of Texas, College Station.

After a period of eight to ten days, it was apparent that the sulfaquinoxaline treated feed was toxic for the birds because egg production declined rapidly, and individual birds became droopy, weak, appeared anemic, and eventually died. All birds that died were examined in the laboratory to determine the cause of death. Tissues for histologic studies were collected and prepared for study by fixation in 10 per cent formalin, embedded in paraffin, cut at 6μ, and stained with hematoxylin and eosin. After removal of the treated feed for a time sufficient for the birds to recover, the program in pen 2 was changed, so that the drug was given in the feed on Monday, Tuesday, and Wednesday of each week rather than continuously.

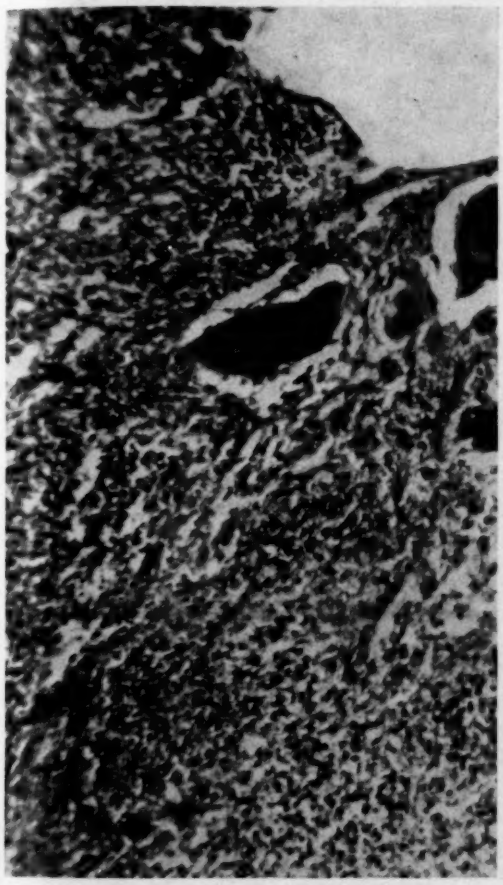

Fig. 2—Lung-nodule with giant cells adjacent to tertiary bronchus. × 175.

The sulfaquinoxaline feeding which was resumed Monday, Oct. 22, 1945, on an intermittent basis, as described, did not cause occult poisoning except when the attendant inadvertently forgot to replace the treated feed on Thursday, Nov. 14, 1945. By Nov. 19, 1945 (table 1), a few birds began to show indications of toxicity to the drug, and some individuals died as a result of it.

DISCUSSION

As seen in table 1, the egg production in pen 2 declined after eight days, and individual birds became droopy, weak, and appeared anemic, following which 13 deaths occurred over a period of two weeks. Losses in egg production and mortality were not experienced to the same extent in pens 1 and 3.

The gross and microscopic examination of typical specimens which died from the effects of sulfaquinoxaline were as follows:

Lung.—While this organ was not frequently involved, typical specimens on gross examination showed numerous greyish white nodular areas ranging from 0.5 mm. to 1.5 mm. in diameter. The appearance of

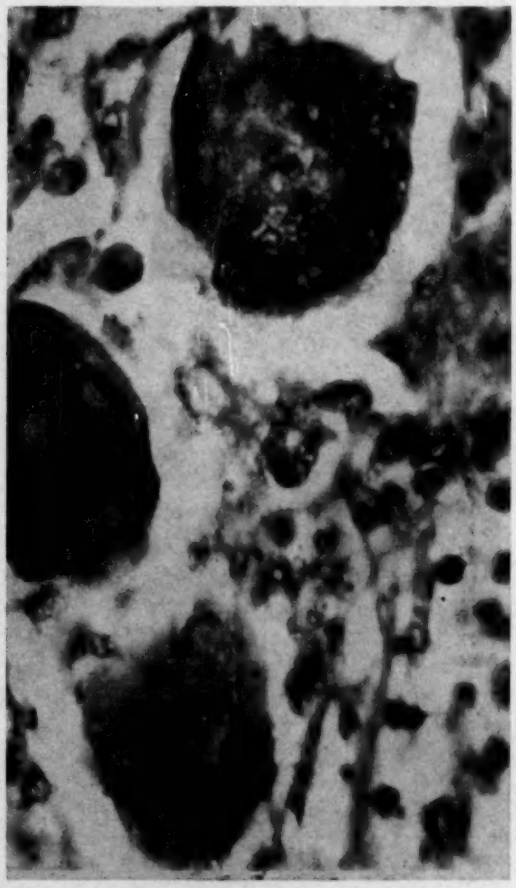

Fig. 3—Lung. Giant cells in lung nodule. × 680.

the lung somewhat resembled that of miliary tuberculosis. On incision, the nodular areas were found to be distributed throughout the tissue. Microscopic examination (fig. 1, 2) showed small rounded areas which resembled tubercles in that many of them were composed of a caseous center surrounded by multinucleated or giant cells. These giant cells varied in size from 30μ to 200μ, or from those containing several visible nuclei up to those with nuclei too numerous to count. The vesicular nuclei of these giant cells were arranged irregularly throughout the cytoplasm (fig. 3). A few of the giant cells were found in the lumina of several tertiary bronchi and in one secondary bronchus. In the center of many of the nodules that had not as yet undergone caseation, eosinophils were fairly numerous. The majority of the nodules were located in the alveolar region adjacent to the tertiary bronchi. At the periphery of the nodular areas, a light leucocytic infiltration composed of lymphocytes and neutrophils was present.

Spleen.—The spleen was the organ most frequently involved, and the lesions grossly resembled those seen in myeloid leucosis. On microscopic examination (fig. 4, 5), nearly every follicle contained multinucleated or giant cells of the type found in the lungs. Caseation necrosis was present in the center of some of these affected follicles. There was only a small amount of lymphoid tissue present in the follicles, and if caseation were not present, the connective tissue stroma was prominent. Some of the follicles also contained eosinophils. A few giant cell collections were seen adjacent to a branch of the splenic artery in a trabecula. The sinusoids were congested. Repeated attempts to isolate bacteria from this organ failed.

TABLE I—Records of Pen 1, 2, and 3 Consisting of 86, 86, and 99 Rhode Island Red Pullets

	Egg Production				Egg Production				Egg Production			
Sept.	Pen 1*	Pen 2**	Pen 3†	Oct.	Pen 1*	Pen 2**	Pen 3†	Nov.	Pen 1*	Pen 2**	Pen 3†
1				1	51 1D	14 1D	63	1	47	45	43
2				2	49	17	60	2	47	52	41
3				3	53	22 1D	61	3	46	45 1I	46
4				4	53	22	59	4	43	46	46
5				5	43	13	63	5	38	50 1D*	46
6				6	50	20	64	6	46	49	51
7				7	53	26	50	7	42	28	46
8	42	58	70	8	50	18 1D*	50	8	42	45	49
9	47	60	71	9	54	26	61	9	35	45	52
10	50	59	63	10	56	19	64	10	30	24	51
11	47	58	69	11	47	25	51	11	43	36	43
12	50	57	71	12	49	22 1D*	64	12	41	46	51
13	50	55 2D	69	13	50	27	61	13	33	32 1D	55
14	49	51	63	14	53	24	53	14	35	35	52
15	50	47	66	15	45	31	55	15	40	39	58
16	53	36	70	16	49	32	44	16	34	36	50
17	54	39	72	17	38	37	50	17	29	28	45
18	50	30	71	18	44	35	55	18	25	30 1D1	56
19	47 1D*	26	69	19	31	38	44	19	26	29	53
20	52	23 1D	71	20	33	36	48	20	32	27 1D	55
21	49	21 1D	65	21	37	37	42	21	36	19	52
22	49	16 1D	74	22	33	33	42	22	26	23 2D	46
23	42	18 1D	73	23	35	39	45	23	30	17 1D	59
24	52	18 3D	73	24	35	43	41	24	33	23	49
25	47	16	70	25	38	42	41	25	30	21 2D	48
26	50	16 1D	74	26	31	38		26	32	15 2D	39
27	50	18	64	27	35	40	45	27			
28	56	19 1D	63	28	39	47	47	28			
29	40 1D*	17	66	29	36	46	47	29			
30	51 1D*	20	77	30	41	49	49 1D*	30			
31				31	36	34	47	31			

*Pen 1 = Unmedicated controls exposed by contact to endemic *P. Avicida.*
**Pen 2 = 0.05 per cent sulfaquinoxaline in feed exposed by contact to endemic *P. avicida.*
†Pen 3 = Controls.
D = died; * = leucosis; 1 = reproductive; I = killed.

Liver.—The liver was frequently involved with greyish white nodules about 1 mm. in diameter scattered throughout the tissue. On microscopic examination (fig. 6), the nodular areas were found to involve the structures of the portal space. These

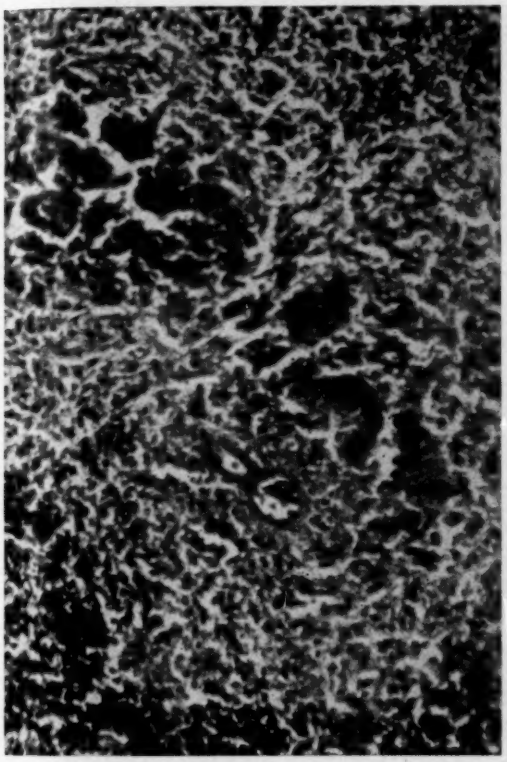

Fig. 4—Spleen. Splenic follicle in lower half of field showing giant cell formation and eosinophilic infiltration. × 175.

nodules had the same appearance as those described in the lung. All of the portal structures in the section were involved. Giant cells were present. Eosinophilic infiltration was greater than in the lungs or spleen. The lymphocytotic and neutrophilic infiltration was of about the same degree as seen in the lungs. Caseation necrosis was present in some of the lesions, and in those not showing caseation, there was a moderate degree of hyperplasia of the connective tissue stroma. The Kupffer cells were prominent in the congested dilated sinusoids. There was no apparent change in the liver cells.

Kidney.—A limited number of scattered greyish white nodules and petechial hemorrhages were present on gross examination. Microscopically, petechial hemorrhages and nodular areas composed of neutrophils, lymphocytes, eosinophils, and giant cells were observed in the interstitial tissue. Cloudy swelling was present in the proximal convoluted tubules. The malpighian bodies showed no apparent change.

Heart.—The heart was occasionally involved and showed a few lesions resembling the nodular structures seen in the other organs. The epicardium and myocardium were both involved.

SUMMARY

Sulfaquinoxaline given in the mash at the rate of 0.05 per cent to Rhode Island Red pullets in egg production (pen 2) resulted in symptoms and death through the effects of the toxicity of the drug. The

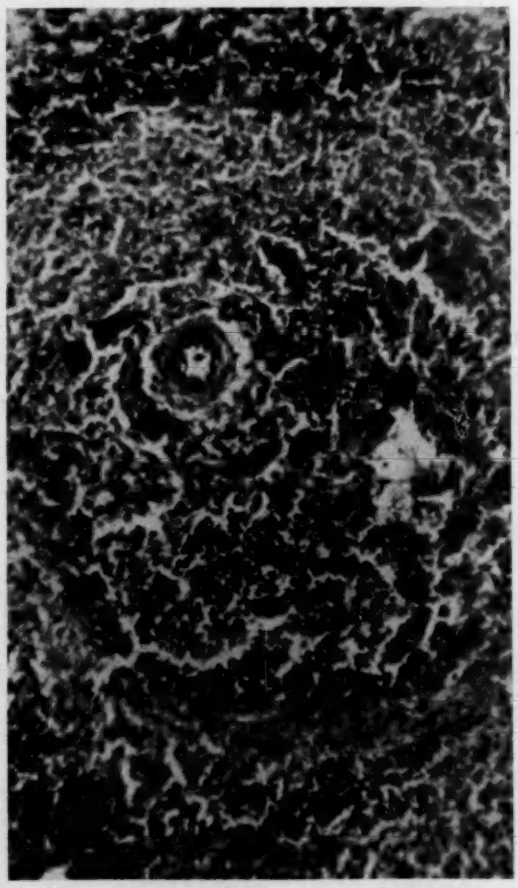

Fig. 5—Spleen. Solitary follicle showing giant cell formation and eosinophilic infiltration. × 175.

intermittent use of the drug on Monday, Tuesday, and Wednesday of each week seemed to prevent the occurrence of poisoning. However, when the attendant forgot to replace the treated mash with regular feed on one occasion, toxic symptoms were again manifested in the birds of pen 2.

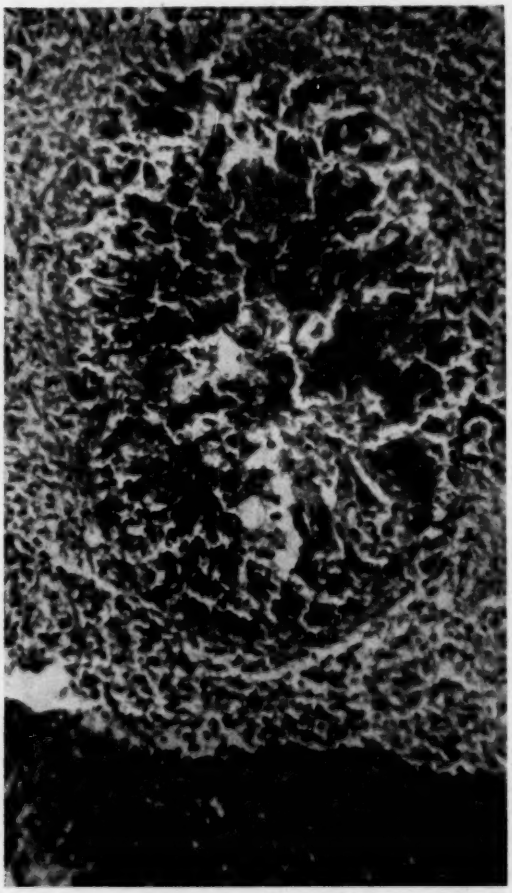

Fig. 6—Nodule in portal canal structure of liver.

The symptoms of sulfaquinoxaline toxicity were characterized by weakness and apparent anemia followed by death in typical cases.

The lesions were characterized by greyish white nodules in the spleen of most birds and in the liver, kidney, heart, and lungs of some specimens. Hemorrhages beneath the skin of the legs and in the combs were observed in most birds that died.

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Use of Antibiotic Agents for Bacterial Sterilization of Respiratory Exudates from Naturally Infected Cases of Newcastle Disease

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IN THE ROUTINE diagnosis of Newcastle disease [avian pneumoencephalitis], we have inoculated embryonated eggs with sterile organ suspensions. Use of this method has avoided the necessity of filtering contaminated exudates. As the virus is not consistently present in any one tissue, it is necessary to inoculate several tissues separately from every suspected outbreak or else pool such tissues for inoculation after each has been proved bacteriologically sterile.

From a study of the incidence of the virus in different organs, our data[1] showed that the virus occurred most consistently in the spleen, that is, in 127 (50%) of the 254 spleens examined. The brain yielded virus in only 20 (19.6%) of the 102 samples examined.

Since the disease is primarily one of the respiratory tract, it seemed logical to assume that filtrates of respiratory tract exudates inoculated into eggs might result in a higher percentage of virus recoveries, sufficiently high perhaps to justify the time and materials required for this technique. However, the results showed that the virus was recovered from only 20 (31.25%) of the 64 filtrates examined. In these tests, the Boerner type of centrifuge filter was used because it particularly suited our needs. The low percentage of virus recoveries was, in part, attributed to retention of the virus by the filters. It was shown, as already reported, that when 1:10, 1:100, 1:1,000, and 1:10,000 dilutions of a Newcastle virus in the form of allantoic fluid were prepared and each was filtered through a different Boerner filter, only the 1:1,000 dilution filtrate was active.

Obvious retention of the virus, by the type of filter used, suggested the use of antibiotic agents as a means of rendering suspensions of respiratory exudates suitable for egg inoculation without the loss of virus incident to filtration.

The use of penicillin and streptomycin separately and combined, for similar purposes, has already been reported by others. Too little is known of the respiratory tract flora of birds; however, Gibbs[2] has found Escherichia of intestinal origin in the respiratory tracts of 33.33 per cent of the birds affected with infectious laryngotracheitis and in 10 per cent of the normal birds as well as staphylococci and other types. Similarly, Bushnell and Brandly[3] recovered such pathogens as *Salmonella pullorum*, *Salmonella gallinarum*, and *Pasteurella avicida*, as well as *Proteus aeruginosa*, a colon type and a member of the Proteus group, from the lungs of chicks showing gasping symptoms. Obviously then, a combination of antibiotics would be required to sterilize the respiratory exudates of birds.

The literature pertinent to the combined use of penicillin and streptomycin for sterilization of exudates and excretions is not too extensive. Rose *et al.*[4] examined 60 samples of human sputum by inoculating each sample into 2 eggs without treatment, 2 treated with 500 units of streptomycin, 2 treated with 500 units of penicillin, and 2 treated with penicillin and streptomycin. Of the 120 embryos inoculated with nontreated sputum, 113 died and all were contaminated. Seven eggs survived five days' incubation but only 3 were sterile. Of the 120 embryos inoculated with penicillin treated material, 72 died of which only 20 were sterile, and 48 survived five days' incubation of which 40 were sterile. Of the 120 embryos inoculated with streptomycin treated material, 60 died and 60 survived five days' incubation. Of the former, 24

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were sterile, and of the latter 36 were sterile. Finally, of the 120 embryos inoculated with penicillin-streptomycin treated material, 43 died of which 28 were sterile, whereas 77 survived five days' incubation and 53 were sterile.

Hodges[5] attempted to recover a virus from the stools of patients affected with epizoötic diarrhea. In these tests, 25 to 200 units of penicillin and 5,000 units of streptomycin, used separately and in combination with 0.2 cc. of a 10 per cent centrifuged stool sample, were inoculated into the amnionic sac of embryonated eggs which were incubated forty hours postinoculation. Of 20 control embryos receiving nontreated stool suspension, all died and were contaminated. Eighteen of the 20 embryos that received the penicillin treated stool suspension died and 2 survived, but all were contaminated. Of the 15 embryos that received streptomycin treated stool suspension, 11 died and 4 survived, but only 1 was contaminated. The death rate was 50 per cent in the 20 embryos that received the penicillin-streptomycin treated stool suspension and only a single embryo was contaminated.

McKee and Hale[6] have reported that nose and throat washings from influenza patients treated with 500 units of penicillin per cubic centimeter, and inoculated into the amnionic sac in a dose of 0.1 cc., permitted only 8 of 24 embryos to survive, but in these the type B virus was demonstrated. However, when 1,000 units of streptomycin were added to the inoculum in addition to penicillin, the embryo mortality was reduced to less than 10 per cent. In addition to the lowered death rate, the authors reported that contamination has been uncommon.

With reference to Newcastle virus, Brandly *et al.*[7] have demonstrated that 1,000 units of penicillin added to the 0.1-cc. dose of virus inoculum in each of ten serial passages in eggs did not affect the titer or infectivity for eggs in any of the egg series, or the end-point infective titer for chickens when compared with the original material. These authors also used penicillin in high concentration to treat contaminated tissues and fecal material intended for egg inoculation, and although the treatment did not completely suppress bacterial growth, virus was frequently demonstrable.

EXPERIMENTAL

Use of antibiotic agents was not anticipated when the first respiratory exudates were suspended for filtration and, consequently, the varying amounts of exudate were brought to a total volume of about 3 cc., an amount insufficient for both filtration and antibiotic treatment studies. Nevertheless, the procedure was followed even after it was decided to treat exudates with antibiotic agents. Thus, the 3-cc. suspension was centrifuged and about 2 cc. of the supernatant passed through a Boerner filter. The approximate 1 cc. remaining was stored in a freezer at —20 C. until 50 selected samples were collected for antibiotic treatment at one time. By this time, the results of egg inoculations with the filtrates and organ suspensions were known.

On March 12, 1947, the approximate 1-cc. volume of each of the 50 selected samples was brought to 3 cc. with broth, centrifuged at 1,500 r.p.m. for ten minutes and 1 cc. of the supernatant placed in each of two sterile tubes. To one tube was added 1 cc. of a solution containing 10,000 units each of penicillin and streptomycin. To the other tube was added 1 cc. of a mixture of tyrothricin, sodium sulfadiazine, and streptomycin. The required 50 cc. of the latter was made by mixing 0.2 cc. of a 2 per cent alcoholic solution of tyrothricin; 2 cc. of a Seitz-filtered solution of sodium sulfadiazine, each cc. of which contained 50 mg. of the agent; 5 cc. of a solution of streptomycin, of which each cc. contained 40,000 units; and 42.8 cc. of water. Thus, each cc. of the above contained 0.08 of 1 mg. of tyrothricin, 2 mg. of sodium sulfadiazine, and 4,000 units of streptomycin.*

The tubes were shaken, incubated at 37 C. for seven hours, and allowed to stand at room temperature for about nine hours. At this time a loopful of material from each tube was streaked on agar and the cultures were incubated for forty-eight hours. No growth occurred in cultures from penicillin-streptomycin treated exudate. Cultures from the same material treated with tyrothricin-sodium sulfadiazine-streptomycin gave growth in only 1 case—four bacterial and one fungus colonies. The mixtures were frozen until inoculated.

On March 13, 1947, duplicate samples were inoculated into 10-day embryonated eggs; on March 20, the remaining 19 duplicate samples

*This is a modification of a formula from Dr. C. A. Brandly who obtained it from Dr. Bernice Eddie.

TABLE I—Results of Egg Inoculation of Organ Suspensions, Filtered and Antibiotic Treated Respiratory Exudates

Group	Case	Bird	Organ Suspensions: Spleen	Brain	Ovary	Oviduct egg	Days from collection to filt.	Filtrate of resp. exudate	Days from collection to treatment	Resp. exud. treated with: PS*	TSdS**
	23830	1	—	—			3	—	38	—	—
		2	—	—			3	—	38	—	—
	23821	1	—	—	—		14	—	28	—	—
1	23861	1	—	—			13	—	23	—	—
		2	—	—			13	—	23	—	—
		3	—	—			13	—	23	—	—
		4	—	—			13	—	23	—	—
	23888	1	—	—	—		9	—	23	—	—
	23886	1	—	—	—		9	—	23	—	—
	23884	1	—	—			9	—	23	—	—
	23760	1	×	—			4	—	45	—	—
		2	×	—			4	—	45	—	—
	23761	1	—	—			3	—			
		2	×	—			3	—			
		3	—	—			3	—			
		4	—	—			3	—	45	—	—
	23778	1	—	—	×		7	—	42	—	—
		2	—	—	—		11	—	42	—	—
	23833	1	×	—	—		10	—			
		2	—	—	—		10	—	31	—	—
2	23844	1	—	—			15	—	25	—	—
		2	—	—			15	—			
		3	×	—			15	—	25	—	—
		4	—	×			15	×			
	23862	1	×	—			9	—	23	—	—
		2	—	—			9	—	23	—	—
	23871	1	×	×			14	—	21	—	—
		2	×	×			14	—	21	—	—
		3	—	×			14	—	28	—	—
	23898	1	—	—	—		12	—	22	—	—
		2	—	—	×		12	—	22	—	—
		3	—	—	—		12	—	22	—	—
	23802	1	—	—	—	×	4	—	32	—	—
		2	—	—	×		4	—	32	—	—
	23774	1	—	—	—	—	4	×	42	—	—
	23781	1	—	—	×	—	10	—	41	—	—
		2	—	—	×	—	6	×	41	×	×
		3	—	—	×		10	×	41	—	—
	23831	1	—	—	×		6	×	34	×	×
		2	—	—	×		6	—	34	—	—
		3	—	—	—		6	—	34	—	—
	23800	1	—	—	—	—	4	—	32	—	—
3		2	—	—	—	—	4	×	32	—	—
	23807	1	—	—	—		10	×	31	—	—
	23834	1	—	—			16	×	30	—	—
		2	×	—			16	×	30	—	—
	23805	1	×	—	×		3	×	31	—	—
		2	—	—	—		7	—	31	—	—
		3	×	—	—		7	—	31	—	—
		1	—	—			12	×	22	—	—
		2	—	—			12	—	22	—	—
	23865	3	—	—			15	×	22	—	—
	23891	1	×	×			8	×	22	—	—
		2	×	×			8	×	22	—	—
		3	—	×			8	—	22	—	—
	23901	1	×	—			7	×	21	—	—

NOTE: Organ suspensions inoculated on the same day as respiratory exudates with the following exceptions; in case 23778, organ suspensions inoculated on seventh day after collection; in case 23781, organ suspensions inoculated on tenth day after collection; in case 23805, organ suspensions inoculated on seventh day after collection excepting spleen and brain of bird 1, which were inoculated on the third day.

× = +.

*PS = penicillin and streptomycin.

**TSdS = tyrothricin, sodium sulfadiazine, and streptomycin.

were inoculated. Three eggs were injected into the allantoic sac with each mixture in a dose of 0.2 cc. per egg. The eggs were incubated seven days postinoculation, at which time all live embryos were discarded. All dead embryos, including most of those dead within the first twenty-four hours, were harvested, cultured, and subinoculations made from them until all embryos of a passage died and showed typical changes, or until all lived, indicating the absence of virus.

The cases selected for the study of the efficiency of filtration of respiratory exudates as compared with the treatment of exudates with antibiotic agents were of three types. The first group included 6 cases with a total of 10 birds. Four cases involved pullets of which 2 showed laryngotracheitis (blood in the trachea) and also a suggestion of Newcastle infection. The other 2 pullet cases as well as 2 cases involving chicks 5 and 6 weeks old, respectively, were clinically typical of Newcastle disease and showed typical autopsy changes. Although virus was not recovered from the filtrates of respiratory exudates or from any organ suspension, these cases were included to determine whether virus could be recovered by antibiotic treatment of exudates. As shown in table 1, in which all the results are listed, antibiotic treated exudates also failed to yield virus.

In the 9 cases involving 24 birds in group 2, one or more tissues in each case had yielded virus, but in no case was the virus recovered from a filtrate of exudate which was also treated with antibiotic agent. Here again, antibiotic treated exudates failed to yield the virus.

The third group included 10 cases involving 22 birds, several of which yielded organs and exudate filtrates that contained the virus. Actually, the 22 birds yielded 14 positive exudate filtrates, as shown in table 1, but exudate from only 2 birds yielded the virus after antibiotic treatment. In both cases, the virus was recovered from penicillin-streptomycin treated exudate as well as from that treated with tyrothricin, sodium sulfadiazine, and streptomycin.

DISCUSSION

Failure to recover virus from a higher percentage of antibiotic treated exudates was probably due to three factors: (1) the filtrations were made four to sixteen days after the exudates were collected, whereas the antibiotic treated exudates were not inoculated until twenty-one to forty-two days after collection. And yet, the two positive results of antibiotic treated exudates were obtained with material that had been held thirty-four and forty-one days, respectively. (2) The antibiotic treated material underwent a further dilution of about 1 to 3. (3) This material was incubated several hours before inoculation, whereas filtrates were inoculated immediately. It is believed, therefore, that had the tests been made simultaneously on comparable dilutions of exudate, a higher percentage of antibiotic treated exudates would have yielded virus.

Admittedly, the dose of antibiotic agents used was probably far in excess of that actually required, but absolute sterility was effected in 99 of the 100 samples as determined by direct aërobic cultures as well as by cultures of all dead embryos harvested.

SUMMARY

1) A mixture of penicillin and streptomycin gave complete bacteriologic sterilization of 50 samples of suspended respiratory exudate treated, and a mixture of tyrothricin, sodium sulfadiazine, and streptomycin sterilized 49 out of the 50 parallel samples treated.

2) Virus was not recovered from ten antibiotic treated samples of respiratory exudate which were also negative on filtration, and which came from clinically suspicious birds whose organs also failed to yield virus.

3) Eighteen samples of respiratory exudate treated with antibiotic agents failed to yield virus as did filtrates of the exudates, in spite of the fact that the virus was recovered from certain organs of these birds.

4) In a final group of 22 birds, the virus was recovered from antibiotic treated exudates from only 2 cases, and, in both instances, from penicillin-streptomycin, as well as from tyrothricin-sodium sulfadiazine-streptomycin treated material. Fourteen of the 22 samples of exudate yielded virus on filtration and, in 4 additional cases, some organ yielded the virus.

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The Use of the Complement Fixation Test in the Detection of Ulcerative Enteritis in Quail

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INFECTIOUS ULCERATIVE enteritis in quail and grouse, or the so-called "quail disease," is one of the most important diseases confronting those engaged in the propagation of upland game birds. The acute form of the disease, following experimental infection, results in death within twenty-four to forty-eight hours. On postmortem examination of the acute cases, the most characteristic lesions are hemorrhagic areas in the intestinal wall. In less acute cases, *i.e.*, those birds dying three or four days after infection, intestinal ulcers of varying sizes are found. In the chronic form, there is marked emaciation and an extensive intestinal ulceration which may extend from the duodenum to the rectum, and, if death is delayed for a sufficiently long time, the intestinal wall may be perforated, resulting in peritonitis. Superficial areas of necrosis are frequently found in the liver. The lungs in some instances may exhibit areas of consolidation.

Epizoötics of quail disease have been known to spread with such rapidity that within three weeks an entire flock of quail is destroyed. The disease may spread in various ways, but the chronic carrier has been established as one of the most important factors in its perpetuation. No method has been available, heretofore, by which the chronic carrier could be detected. This paper describes a complement fixation test which may be used to detect carriers of quail disease.

The serums used in these tests were obtained from birds suffering from artificially induced quail disease and also collected from experimental birds at the Refuge, where several birds dying of the disease had been found. Serums from birds known to have never come in contact with quail disease were used as controls.

Many attempts were made to prepare a suitable antigen. Of these, the one showing the greatest specificity, the lowest anticomplementary properties, and the widest range was prepared from the livers and hearts of birds dying of quail disease. One hundred grams of finely cut liver and heart tissues, taken from birds showing the lesions of chronic quail disease, were placed in a flask with 125 cc. of 95 per cent ethanol to coagulate the tissue proteins. After five days of such exposure, the alcohol was filtered off, and the tissues placed in an incubator at 37 C. for twenty-four hours to dry. The acetone soluble fractions were extracted from the dried tissues by adding 200 cc. of chemically pure acetone and extracting for one week, after which the acetone was filtered off, and 200 cc. of fresh acetone was added. At the end of twenty-four hours, the acetone was filtered off, and the tissues were placed in the incubator to dry. The dried tissues were ground in a mortar, placed in a flask, and 200 cc. of 95 per cent ethanol was added. The tissues, extracted for ten days at room temperature, were shaken for five minutes or so each day during the extraction. At the end of this time, the alcoholic extract was filtered off, placed in a brown glass bottle, and stored at room temperature for later use as antigen.

The antigen was titrated with a serum from a bird known to have a chronic case of quail disease. Chronicity was determined by postmortem examination of a bird previously infected. The blood was collected by decapitation of the sick bird. If lesions of a chronic state of the disease were present,

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serum from the bird was used as a positive serum.

The usual hemolytic system consisting of sheep cells, guinea pig complement, and rabbit antisheep cell amboceptor was employed. The amboceptor was so diluted that 0.05 cc. contained one hemolytic unit. The unit for the purpose of these experiments was considered to be the smallest amount of hemolysin which gave complete hemolysis

TABLE 1

Artificially infected birds		Nonexposed birds	
Bird (No.)	Test reading	Bird (No.)	Test reading
3036	2+	3286	—
3055	3+	3273	—
3035	2+	3236	—
3058	4+	3290	—
3084	2+	3295	—
2761	2+	3271	—

of 0.1 cc. of a 5 per cent suspension of sheep erythrocytes, with an excess of complement in the water bath at 37 C., at the end of forty-five minutes. Equal amounts of diluted amboceptor and a 5 per cent suspension of sheep cells were mixed together. Fresh complement or complement preserved by lyophilization was titrated on the day of the test.

In the test, two tubes were set up for each serum. The first tube received 0.04 cc. of undiluted, unheated serum, and the second 0.02 cc. The serums were not inactivated by heat, since it was found that such inactivation produced negative results even with known positive serums. A suitable antigen dilution was added in 0.1-cc. amounts. Complement was diluted to contain 2 units in 0.1 cc. Fixation was carried out in the ice box for eighteen to twenty hours after which 0.2 cc. of sensitized sheep cells was added to each tube. The tubes were then incubated for thirty minutes at 37 C. in the water bath. Controls were run to determine the anticomplementary action of the serums, the anticomplementary action of the antigen, and the presence of native sheep hemolysin in the serums.

In one series of tests, 6 birds were infected artificially by feeding. Ten days later they were bled, and the serums tested for the presence of complement fixing antibodies. Nonexposed birds were run as controls. The results of one test series are shown in table 1.

Tests were run on 100 artificially infected birds, and of these, 97 reacted positively. Of the 50 nonexposed birds used as controls, 5 gave weak positive reactions. These positive reactions were attributed to hemolytic products present in the test serums, as none of the 5 birds showed lesions on postmortem examination.

During the course of these experiments, an outbreak of ulcerative enteritis occurred in a flock of quail on nutrition experiments on another farm at the Refuge. The 403 birds constituting the experiment group were tested for complement fixing antibody. Of these, 46 reacted positively to the test. These 46 birds were held in isolation, and 9 of these died of quail disease. No deaths attributable to quail disease occurred in the birds reacting negatively to the test.

SUMMARY

A modified complement fixation test using tissue extract for an antigen for the detection of infectious enteritis in quail is described.

Results of experimental trials and of tests of birds in a spontaneous outbreak of ulcerative enteritis in a flock of 403 quail are given.

The results obtained in the tests indicate that the complement fixation test may be employed in the detection of carriers of ulcerative enteritis in quail.

A Comparative Study of the Use-Dilution Method and the F.D.A. Phenol Coefficient Method Applied to Some Veterinary Disinfectants

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SINCE it was observed that various chemical agents had either an inhibitory effect on the multiplication of bacteria or a lethal action, many laboratory methods have been devised to find a satisfactory means of measurement indicative of practical application. Many of the proposed methods of germicidal assay sort chemicals into lethal and nonlethal groups, and some sort lethal compounds by degree of activity on a laboratory basis. Most of the methods fail to demonstrate the dosage of the compound that can be applied successfully to field use. Because of the multiplicity of uses, with corresponding variables in interferences to action, it is probably impossible to find any one method, applicable to all practical uses, which will measure concentrations of disinfectants. For example, it has been demonstrated that the speed of kill varies widely in various types of chemicals. Mercurials, such as mercuric chloride, coat the bacterial cell wall with mercury ions which, if left in contact for relatively long periods of time, will ultimately destroy the cell. This has been definitely proved by the use of appropriate neutralizing agents. It can be demonstrated that mercuric chloride in relatively high concentrations does not kill test organisms in ten minutes. It does not follow that such information proves the ineffectiveness of this compound. It does prove that, where rapid kill is necessary, or where neutralizing agents follow the application of the disinfectant before kill has been attained, this compound would have little value. Where prolonged exposure occurs and no neutralizing agents intervene, disinfection by this compound would be perfectly satisfactory. In the developments of a laboratory procedure for the evaluation of disinfecting agents, the laboratorian should take into consideration the results attained in the field in the control of disease organisms. It has been conclusively proved that chlorine, when applied to properly clarified water, which had been contaminated previously by sewage, destroys any typhoid bacilli that have passed through the clarification system. This is proved by checking the incidence of typhoid fever in communities that, prior to chlorine treatment, had a high incidence of waterborne typhoid fever. Trick laboratory methods of testing the germicidal activity of chlorine, which show a failure to kill in concentrations now routinely applied in water treatment, would be of no practical value. Field experiences in all applications of disinfection should be studied fully in the interpretation of laboratory methods of measuring disinfectants.

TABLE I—Resistance of Test Organisms to Drying on Glass Test Rods

Drying time	Bacteria surviving drying	
	Rod 1	Rod 2
Eberthella typhosa		
20 minutes	243,000	292,000
30 minutes	112,000	288,000
60 minutes	564,000	475,000
90 minutes	487,000	110,000
120 minutes	39,000	20,000
Staphylococcus aureus		
30 minutes	1,130,000	1,190,000
60 minutes	1,120,000	1,060,000
90 minutes	1,300,000	1,430,000
120 minutes	1,480,000	1,550,000
150 minutes	1,090,000	1,330,000

This paper presents a new technique of measuring disinfectants, designed to approach practical application more closely than does the present F.D.A. phenol coefficient procedure. The term "disinfectant" is used in this paper to cover chemical agents used for the control of disease-producing bacteria on inanimate surfaces.

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The method was not designed to measure the activity of chemical agents for controlling bacteria on skin or in wounds.

Ever since Koch, in 1881, first presented his famous thread method of testing disinfectants, research workers have been devising new methods or adaptations of old procedures, always with the objective of finding a more satisfactory method of evaluating chemical disinfecting agents for regulatory purposes or practical application. It would add little to this paper to review the many methods devised. Suffice it to state that none have presented a satisfactory method.

TABLE 2—Data Sheet on B.T.C. Using the Use-Dilution Technique with Staphylococcus Aureus as Test Organism

	Period of exposure in minutes							
	1		5		10		30	
Dilution	Growth in broth	No. of bacteria	Growth in broth	No. of bacteria	Growth in broth	No. of bacteria	Growth in broth	No. of bacteria
1:5,000	+	0	—	0	—	0	—	0
	+	0	+	0	—	0	—	0
1:10,000	+	1000	—	0	+	0	—	0
	+	1400	+	0	+	0	—	0
1:15,000	+	3100	+	0	+	0	—	0
	+	2900	+	10	+	0	—	0

Number of bacteria on control rods 1,460,000.

LITERATURE CITED

It is hard to evaluate disinfectants on a comparative basis because we have no single reliable method of comparative testing. The F.D.A. phenol coefficient indubitably gives comparable results when testing coal-tar products, but it can not be used to compare the disinfecting value of a quaternary ammonium salt, a mercurial, and a chlorophenol. Tilley[1] has stated that "a phenol coefficient has no practical value whatever, except as a means of preventing the use of positively worthless preparations and as an aid to the manufacturer in maintaining the uniformity of his product." Varley and Reddish[2] have stated that "the phenol coefficient figure is a suitable measure for the practical value of phenol-like disinfectants when the factor 'twenty times the phenol coefficient' is employed for calculating the dilution for use in practice." However, since the manufacturer usually recommends a dilution of his product, this recommended dilution should be used in evaluating the disinfectant rather than the killing dilution obtained in the phenol coefficient. Also, this dilution should be based on definite tests against the organism it is desired to kill in practical use. It is unfair to classify a disinfectant as poor merely because its F.D.A. phenol coefficient value is low. At the recommended dilution, it may readily kill the desired organism in a time comparable to that in actual use. If the test organisms are not affected at this dilution, a new use dilution should be recommended.

Hyma[3] has demonstrated that the dilution coefficient should be taken into consideration when evaluating a disinfectant by its phenol coefficient. Upon diluting a disinfectant to one-half its original concentration, the disinfecting value may be reduced from two to 64 times depending upon its dilution coefficient. This factor of dilution could be eliminated if the disinfectant was evaluated at the manufacturer's recommended dilution. The need of a use dilution was suggested by Mallmann[4].

The use-dilution method proposed by Mallmann[5] for the testing of disinfectants was devised in an effort to improve upon the F.D.A. phenol coefficient method and to evaluate disinfectants under conditions more comparable to those under which they are to be used. In disinfection, the object to be treated is frequently covered with layers of dried organisms. The glass rod used in the use-dilution technique is also covered with layers of dried organisms and, therefore, would more nearly simulate actual use conditions. The glass rods are rinsed before they are put into the culture medium, so there should be less carrying over of disinfectant than there is in the phenol coefficient procedure.

TABLE 3—Data Sheet on Aseptogen Using the Use-Dilution Technique with Staphylococcus Aureus as Test Organism

	Period of exposure in minutes							
	1		5		10		30	
Dilution	Growth in broth	No. of bacteria	Growth in broth	No. of bacteria	Growth in broth	No. of bacteria	Growth in broth	No. of bacteria
1:5	+	600	+	70	+	0	—	0
	+	2800	+	60	+	10	—	0
1:10	+	1400	+	130	+	40	—	0
	+	3700	+	150	+	10	—	0
1:20	+	6000	+	1700	+	120	+	0
	+	4500	+	1300	+	100	+	20
1:40	+	7500	+	1600	+	630	+	90
	+	8000	+	2000	+	500	+	70

Number of bacteria on control rods 463,000.

A comparative study of the F.D.A. phenol coefficient method and the use-dilution technique was undertaken. Common disinfectants, many employed in the veterinary field, were used for two reasons: (1) because most of them have a relatively low phenol coefficient which means that they have a smaller dilution range in which to work efficiently; (2) because veterinarians depend a great deal on disinfectants, and it was desired to know

whether these compounds are effective at the recommended dilution. A few quaternary ammonium compounds were included to obtain a picture of disinfectants with high phenol coefficients.

EXPERIMENTAL PROCEDURE

The test organisms used were a standard strain of *Eberthella typhosa* and *Staphylococcus aureus* obtained from the U. S. Food and Drug Administration.

Eberthella typhosa was grown in standard broth,[6] and *Sta. aureus* was grown in Difco disinfectant testing broth (experimental). The 24-hour subculture of *Sta. aureus*, used for test purposes, was grown in F.D.A. broth. The Difco disinfectant testing broth (experimental) was used for carrying the culture by daily transfers because the organism retained its resistance to phenol more closely than when it was carried on F.D.A. broth. The test cultures were transferred daily and fresh transfers were made monthly from stock agar slant cultures.

In the use-dilution method 22- to 26-hour broth cultures of *Sta. aureus* and *E. typhosa* were shaken for fifteen minutes to break up clumps. Sterile glass rods, 1 in. in length and ¼ in. in diameter having a loop at one end for handling, were dipped into the broth culture and then carefully laid for drying on sterile filter paper in Petri dishes, care being exercised to avoid rolling while drying. Because only partial drying was found necessary, the drying was done at room temperature. A drying period of thirty minutes was used.

Medication pots (1 in. by 3 in.) containing 10 cc. of the test disinfectant and the pots containing the physiologic saline rinse (10 cc.) were placed in a 20 C. water bath. The bath was held at this temperature throughout the test.

At the end of the drying period, four rods were dropped simultaneously into each medication pot containing the test disinfectant. At intervals of one, five, ten, and thirty minutes, a rod was removed and immersed for one minute in a tube containing 10 cc. of sterile physiologic saline. It was then placed in a tube containing 10 cc. of nutrient broth. This tube was shaken vigorously to remove all organisms adhering to the rods, and suitable dilutions were plated in plain nutrient agar to measure quantitatively the extent of kill. The tubes containing the rods and the plates were incubated at 37 C. for forty-eight hours. Lack of growth at the end of this period of incubation was accepted as evidence of complete kill.

Controls were run by dropping rods covered with the dried organisms into the rinse tubes of sterile physiologic saline for one minute and then into the tubes containing 10 cc. of broth. Dilutions of 1:1,000 and 1:10,000 were plated. The count was recorded as the total number of organisms washed off the rod in nutrient broth. The organisms were checked for standard resistance by following the F.D.A. phenol coefficient procedure.

The use dilutions reported in this paper are the minimum concentrations that killed completely in ten minutes but not in five minutes.

After obtaining some negative controls when *E. typhosa* was used, it was decided to check the effect of drying time on the viability of the test organisms. Inoculated rods were dried for varying lengths of time and then were dropped into 10 cc. of physiologic saline for one minute and then into 10 cc. of nutrient broth. Suitable dilutions were plated. The results are presented in table 1. An examination of the data shows that *Sta. aureus* is resistant to drying over a period of 150 minutes whereas *E. typhosa* showed a marked reduction after ninety minutes. In earlier tests, it was noted that the resistance to drying of *E. typhosa* varied considerably from time to time. Since preliminary tests showed a reduction after thirty minutes' drying, it was decided to use a period of twenty minutes for subsequent tests. Although data are presented for the use-dilution technique, using *E. typhosa* to show relative values, the writers

TABLE 4—Comparative Data on a Group of Phenol-like Compounds, Using the F.D.A. Phenol Coefficient Procedure and the Use-Dilution Technique

Name of product	Manufacturer's recommended use dilution	*Eberthella typhosa*		*Staphyloccus aureus*	
		FDA procedure killing dilution	Use dilution	FDA procedure killing dilution	Use dilution
Phenol	1:20	1:90	1:110	1:60	>1:70< 1:80
Amphyl	1:160	1:850	>1:700< 1:800	1:250	1:100
Aseptogen	1:20	1:150	>1:200< 1:300	1:10	1:5
Phenolor	1:30	1:300	1:400	1:150	1:100
Antiseptine	1:40	1:350	1:400	1:60	1:50
Lysol	1:100	1:600	1:600	>1:150< 1:200	1:200
Creolin	1:80	>1:800< 1:900	1:900	>1:50 < 1:75	1:40
Dr. Hess Dip...	1:70	1:400	1:400	1:60	1:25
Liquor cresolis saponatus	1:50	1:150	1:200	1:180	>1:50< 1:75
Zenoleum	1:50	1:350	>1:300< 1:400	>1:20 < 1:30	1:20

believe that the use-dilution technique for routine use should be limited to tests with *Sta. aureus*.

Several data sheets (table 2 and 3) on the use dilution, using *Sta. aureus* as a test organism, are presented. These are included to show the information obtained by this technique. Table 2 presents the data obtained with a quaternary ammonium compound (B.T.C.). The dilutions are based on the anhydrous compound. It will be observed that complete kill was obtained in ten minutes in a dilution of 1:5,000, although only one tube out of the two showed growth at 1:5,000 after five minutes exposure Plate counts were negative even after one-minute exposure at 1:5,000 dilution.

The fact that the numbers of colonies appearing on the plates in the 1:10,000 and 1:15,000 dilutions in one minute are low cannot be charged to bacteriostatic activity, as longer exposures failed to show any visible organisms. Although the broth tubes show growth at all dilutions after an exposure of one minute, the total number of visible organisms at a dilution of 1:15,000 in one minute is less than 0.01 per cent of the number found on the control rods. The speed of reaction shown by this compound is in keeping with studies on speed reaction techniques presented by Mallmann, Kivela, and Turney[7]. It is also interesting to note that few organisms survived, as shown by the fact that growth occurred in the broth tubes in dilutions of 1:10,000 in ten minutes.

These data show clearly that when complete kill is used as a measurement of the effectiveness of a disinfectant, an answer is obtained which may be out of line in regard to the use of the compound as an aid in the lessening of disease hazard. Percentage kill can be misleading, but certainly it is true that where 99.99 per cent of the bacteria are destroyed in a dilution of 1:15,000 in one minute, with extremely high bacterial population, the product has merit.

Table 3 presents the data obtained with Aseptogen, using the use-dilution technique. This product failed to show complete kill in five minutes in a dilution of 1:5, basing the dilution on the product figured as 100 per cent. The compound does have germicidal properties as shown by the fact that complete kill was obtained in a dilution of 1:10 in thirty minutes. This compound gave a kill of 99.98 per cent in one minute in a dilution of 1:40. The residual viable cells left after one minute exposure die very slowly, but a continued kill is shown in that the count dropped from approximately 8,000 after one minute exposure to 80 in thirty minutes. If we are to accept the manufacturer's recommended dilution of 1:20, we find that the compound fails to kill completely even after thirty minutes exposure. However, if we accept percentage kill, the compound effected a 99.99 per cent kill in one minute.

TABLE 5—Comparative Data on a Group of Phenolic Derivatives Using the F.D.A. Phenol Coefficient Procedure and Use-Dilution Technique

Name of product	Manufacturer's recommended use dilution	*Eberthella typhosa*		*Staphyloccus aureus*	
		FDA procedure killing dilution	Use dilution	FDA procedure killing dilution	Use dilution
Benchlophen	1:200	1:200	>1:150< 1:200	1:1200	1:50
Dowicide	.....	1:5000	1:5000	>1:1000< 1:5000	1:750

The first series of compounds tested was phenolic or cresylic type compounds. No attempt was made to collect any comprehensive representation of this group of compounds which is on the market. Most of the products were in the laboratory, but a few, such as Aseptogen, Dr. Hess Dip, and Zenoleum, were purchased on the local market. The list of compounds reported in table 4 is fairly representative of those commonly used. The comparative data on this group of compounds, using the F.D.A. phenol coefficient procedure and the use-dilution technique, are presented in table 4. Both *E. typhosa* and *Sta. aureus* were used as test organisms. When *E. typhosa* was used as the test organism, the killing dilution, using the F.D.A. technique, was approximately the same dilution as that obtained with the use-dilution procedure. It will be

TABLE 6—Comparative Data on a Group of Quaternary Ammonium Compounds Using the FDA Phenol Coefficient Procedure and the Use-Dilution Technique

Name of product	Manufacturer's recommended use dilution	*Eberthella typhosa*		*Staphyloccus aureus*	
		FDA procedure killing dilution	Use dilution	FDA procedure killing dilution	Use dilution
B.T.C.	1:6400	1:18,000 1:20,000	1:15,000 1:20,000	1:20,000	1:5,000
Tetrasan	.....	1:60,000	1:10,000 1:20,000	1:35,000	1:10,000

observed that the manufacturer's recommended use dilution is generally less than one-fifth that of the killing dilution.

When *Sta. aureus* was used as the test organism, the use dilution was generally slightly lower than the killing dilution obtained by the F.D.A. procedure. In many cases the use-dilution technique gave a killing dilution less than the use dilution recommended by the manufacturer. In the case of Zenoleum, Dr. Hess Dip, and Creolin, the F.D.A. procedure killing dilution was below the manufacturer's recommended dilution.

Inasmuch as the disinfectants are used by the veterinarian and others to kill all types of pathogenic bacteria (gram-positive and negative), the fact that the manufacturer's recommended dilutions fail to kill *Sta. aureus* completely would either invalidate their use or suggest that stronger dilutions be used.

These data show the fallacy of using *E. typhosa* as a measurement of the effectiveness of the phenolic disinfectants and the rule of 20 times the phenol coefficient for calculating the manufacturer's recommended dilution for field use.

In the second series of tests, two chlorophenol compounds were selected, namely, Benchlophen and Dowicide C. The latter was selected because it represents a phenolic type product with a relatively high phenol coefficient. The results are presented in table 5. An examination of the data shows that, using *E. typhosa*, the F.D.A. technique killing dilution and the use dilution are the same; however, when *Sta. aureus* was used, a marked difference occurred in the killing dilutions of the two techniques. Benchlophen showed a killing dilution of 1:200 using F.D.A. technique but a use dilution of 1:50. The latter is only one-fourth that recommended by the manufacturer.

In the third series of tests are included two representatives of the quaternary ammonium compounds. These are included because they show very high phenol coefficients. The data are presented in table 6. The results using *E. typhosa* give comparable results by both methods on B.T.C., but the use dilution on Tetrasan was much lower than the F.D.A. killing dilution. When *Sta. aureus* was used as a test organism, the use dilution was much lower than that of the F.D.A. technique killing dilution.

SUMMARY

The use-dilution technique offers a new means of measuring more dependably the actual dilution of disinfectants for field use. This is particularly true of disinfectants which exhibit high phenol coefficients.

Data are presented on a number of compounds used in the veterinary field showing the comparative dilutions recommended by the manufacturer and those obtained by the use-dilution technique.

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Observations on the Life History of Lung Nematodes Using Snails as Intermediate Hosts

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DUE TO THE excellent experimental work of A. Hobmaier and M. Hobmaier,[1,2,3,4,6] the life cycles of several species of Metastrongylus are known in detail. The peculiarities discovered by these investigators have been observed in different genera, and they are most probably common to the life histories of most genera of this group of nematodes. The present author has become convinced of this point through investigating the life histories of five Palestinian species: *Aelurostrongylus abstrusus* (Raillet, 1898), Cameron, 1927; *Muellerius capillaris* (Mueller, 1889), Cameron, 1927; *Protostrongylus kochi* (Schuls Orlov and Kutass, 1933), Böhm and Gebauer, 1934; *Cystocaulus ocreatus* (Raillet and Henry, 1907), Micacic, 1939; and Troglostrongylus.* It should be noted that the life histories of the three first-named species have been the subject of investigation by several workers. The present chapter, based on the personal experience of the author, gives a comparative summary of observations. Details concerning particular species will be published later.

The life cycles of all the five above mentioned species follow the same general pattern. There are, however, differences between them in the initial stages of larval development. In Troglostrongylus sp., in contrast to the other four species, the eggs when laid already contain fully developed first-stage larvae. In order to secure these larvae for experiments, the eggs should be kept in water or saline for one day, when hatching occurs. In the other species freshly laid eggs contain only initial embryonal stages, and the development of the larva within the egg can take place only in the parenchyma of the lung of the host. If eggs of these species are taken from gravid females or from the tissue of the host in an undeveloped state, the larvae will not develop *in vitro*. Therefore, hatched larvae removed from the lungs of the host were used for life history experiments.

EXPERIMENTAL METHODS AND OBSERVATIONS

The following technique was employed to secure these larvae. The cut surface of infected part of the lung was scraped with a scalpel, and the macerated material was collected in saline. The tissue was then screened through No. 25 brass mesh, which retained the coarse particles but not the larvae. The filtrate was washed several times by centrifugation in fresh saline. Infection of the snails was carried out by two methods: (1) The snails were permitted to crawl about for approximately fifteen minutes in a Petri dish containing fluid rich in larvae; (2) a drop of fluid containing the larvae was introduced into the opening of the shell into which the snail had withdrawn, and when the moisture had caused the snail to extend its foot, a few additional drops were placed on it. Snails moistened in this manner were kept extended for several minutes. Although both methods are effective, it has been found that the latter is surer and invariably produces a more intensive infection. Numerous snails were employed in every experiment and, in order to follow the development of the larvae, specimens were taken at intervals, and their tissues, in particular the foot, were examined microscopically after having been compressed between two slides. In all cases, batches of noninfected snails of the same species were kept under identical conditions as controls.

The following species of snails and slugs were used in the experiments: *Chondrulla septemdentata, Helicella barbesiana, Helicella vestalis joppensis, Helix cavata, Levantina caesareana, Levantina hierosolyma, Monacha syriaca, Retinella nittellina, Theba pisana,* Agriolimax sp., and *Limax flavus.*

Our observations led us to conclude that the larvae of metastrongylids requiring land

This study was conducted in the Department of Parasitology, The Hebrew University, Jerusalem, Palestine.

The author is from the Government Central Laboratories, Jerusalem.

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*This species is regarded as new. It has been tentatively named *Troglostrongylus brevior* sp. nov. A description will be given later.

snails as intermediate hosts pass through four stages in their development (before reaching the final host), and not through three as is generally accepted. The first-stage larva develops in the lung of the definitive host and the other three in the snail. Only two molts occur during this development, between the first and the second stage and between the second and third stage.

The first-stage larva is usually very active and when in contact with the skin of the snail makes vigorous efforts to penetrate it. During the first few days after penetration, the larva remains unchanged in the tissues of the snail's foot. Eventually development becomes evident, the larva growing longer and wider, and food granules appearing in its intestinal cells. After the larva has reached a certain limiting size, peculiar to each species, its activity slows down, and it molts without shedding the old cuticle, thus passing into the second stage. When put into water in this stage, the larva moves slowly inside its sheath only. After a comparatively short period during which practically no change occurs in the larva, its activity ceases entirely, and the second molt occurs. The larva is now in its third or preinfective stage. It is longer but slimmer than the second stage larva, contains fewer food granules, and is encased in two sheaths. It becomes more active, and when put into water it coils and uncoils vigorously but practically without progressive motion. The larva remains in this stage for a comparatively short period and is transformed, within its double sheath, into the last or infective stage.

It may be pointed out that the differentiation between the preinfective and infective stages has not been made by earlier investigators. At the most, a suggestion of it may be seen in the observations made by Hobmaier[5], who exposed the tissues of infected snails to artificial digestion, as a result of which some larvae (of *Crenosoma mephitidis*) appeared in the sediment encased in two sheaths and dead, while others were found unsheathed and alive. Hobmaier and others have assumed that the former were larvae which had not yet attained full development, and which were unable to withstand the action of digestion. According to the present author's interpretation, however, the dead larvae were in the preinfective, while the living ones were in the infective stage.

Although there is no molt between the third and the infective stages, the difference between them is obvious. The physical properties of the sheaths undergo changes: in the third stage they are elastic and strong and do not break down when the larvae are pressed out of the tissues of the snail. The sheaths of the infective stage, on the other hand, are very fragile, and they break easily when pressure is applied. Apparently they are also more easily digestible, as may be concluded from the previously mentioned experiment of Hobmaier. Furthermore, the structure of the esophagus changes. In the third stage numerous nuclei are seen distributed over its entire length, while in the infective stage they disappear and coarse granules fill the posterior portion of the esophagus. In the intestine of the third-stage larvae, food granules are invariably seen, though they are less numerous than in the second stage, whereas in the infective stage they disappear almost completely. The third-stage larvae are sluggish in movement even if artificially freed of the sheaths. The infective larvae, by contrast, move vigorously, exceeding even the first-stage larvae in activity. They move not only in water but even in the tissue of the snail when the sheaths have been ruptured through compression. Such movement is never observed in other stages.

The course of development of each species of Metastrongylus in the snail has certain specific limits which depend on the individual properties of the larvae, on the appropriateness of the snail species as a host, and on environmental factors.

Larvae of the several stages of development occurred side by side for some time after artificial infection, even when the snails were kept under optimum conditions. After a suitable period, however, all larvae eventually reach the infective stage. This factor of aptitude of individual larvae for rapid or slow development has been manifested in all species studied by the author, and it probably occurs among all Metastrongylus nematodes.

Infection experiments performed with the above mentioned species of Metastrongylus and snails have demonstrated that the larvae of all the given nematode species (except *P. kochi*) developed in almost all the snail species studied. This apparent lack of specificity of these nematode species with respect to their intermediate hosts is, however, not absolute. Although many species of snails may serve as intermediate hosts to any one of the four particular nematode species studied, their capacity for supporting the development of the larvae is different. Thus, the intensity of infection and the pace of development is different in various snails kept under identical conditions. With respect to these properties, it was possible to divide the snail hosts into two categories: "appropriate" and "inappropriate." In species of the first category, infection invariably succeeds, many larvae may develop in each individual, and the development is comparatively rapid. In the snails of the inappropriate category, only a small percentage of individuals become infected, for

only a few larvae succeed in penetrating the skin of the snail and development proceeds slowly. This differentiation is, however, not sharp, and intermediate degrees of appropriateness are observed in some snail species.

Among the environmental factors affecting the development of metastrongylid larvae in snails, the most important is temperature. Other factors, for instance, the degree of humidity or the feeding conditions, play comparatively insignificant rôles. Unfortunately, observations concerning this phenomenon are practically lacking in the literature, and the conclusions expressed here are based only on the study of Palestinian species in the range of temperature between 4 C. and 30 C. It has been observed that the development is quickest at approximately 30 C. and that it slows down at lower temperatures. The lowest developmental threshold has not been established for each of the nematode species studied. It has been found, however, that the minimum temperature at which development will proceed varies with the species studied. Marked differences have been observed when the infected snails of the "appropriate" category have been kept in the refrigerator at 4 to 8 C. Under these conditions the larvae of *P. kochi* and *A. abstrusus* had undergone no perceptible development for periods of five and seven months, respectively, while they require only forty-six to forty-nine days and nineteen to twenty days, respectively, at optimum temperature. *Cystocaulus ocreatus* had undergone only initial stages of development in the refrigerator, not reaching the first ecdysis even after five months, while it requires as little as seventeen to eighteen days for full development at optimum temperature. Refrigerated *M. capillaris* reached the infective stage only after five months, as against thirty-four to thirty-five days at optimum temperature, and refrigerated Troglostrongylus reached the infective stage after forty days, as against eight days at optimum temperature.

The retardation of development at lower temperature does not involve diminution of viability or of the developmental capacity of the larvae, as may be seen from the following experiment. Snails of the appropriate category were infected with larvae of all five species of nematodes and were immediately put into the refrigerator. They were left at this temperature for varying periods during which no development occurred. Thus, *P. kochi* was refrigerated for fifty days or for 120 days. *A. abstrusus* for 120 days, *C. ocreatus* for fifty days, *M. capillaris* for fifty days, and Troglostrongylus sp. for ten days. After these refrigeration periods, some of the snails were transferred to optimum temperatures. In all cases development of the larvae began at once and was completed after the period required by snails kept only at optimum temperatures. The control snails left in the refrigerator exhibited either a retardation or no development (depending on the species), during the same period of time.

RESULTS

The results of these experiments offer an explanation for observations previously made on the development of Metastrongylus larvae in snails kept outdoors. In our preliminary experiments, snails artificially infected with larvae of *C. ocreatus* and *M. capillaris* were left in jars outdoors from December to April (during which time the average daily temperature fluctuated between 3.9 C. and 18.7 C.) in order to keep them under conditions resembling those in nature. From December to January (4.8 C. to 12.5 C.), practically no development of the larvae could be observed. Initial changes occurred in February (3.9 C. to 9.5 C.), and development was completed by the end of April. It is evident that under natural conditions the development of Metastrongylus larvae in snails is retarded during the cold winter months. This phenomenon has far reaching epidemiologic significance, and it will be discussed in a paper dealing with the bionomics of metastrongylids of sheep.

Examination of naturally infected snails collected in a field heavily polluted with sheep excrement in the vicinity of Jerusalem revealed multiple infections with two or three species of Metastrongylus larvae of the sheep parasites, *C. ocreatus, M. capillaris,* and *P. kochi.* This observation raised the question whether simultaneous infection of the snail host with another species influences the development of any particular nematode. For this purpose snails were artificially infected simultaneously with the above mentioned species and were kept thereafter under optimum conditions. Subsequent examination of the snails showed that each nematode species developed as if it were alone, *i.e.,* that the larvae of any of these species reached their infective stages during the periods usually required by them when no other larvae were present. It is probable that the same phenomenon